Regulation of mRNA Stability via BRF1 and other AU-Binding Proteins

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For Claudia, who constantly stayed at my side during these "turnover-years".

Summary

Steady state levels of mRNAs are determined by the rate of synthesis and degradation. A well-known *cis*-element conferring instability to mRNA is the so-called AU-rich element (ARE), which is present in the 3' untranslated region (3'UTR) of many cytokines, chemokines, growth factors or proto-oncogenes. The ARE is recognized by a variety of ARE-binding proteins (AUBPs), which decide about the fate of the RNA. Multiple signaling cascades regulate the activity of the AUBPs. Butyrate response factor 1 (BRF1), a Tis11 protein family member, was functionally cloned in our lab, as an ARE-mRNA destabilizing protein. However, not much is known about the mode of action of this protein and its physiological role.

This thesis deals in a first part with the regulation of BRF1. Analysis of BRF1 protein sequence revealed multiple putative phosphorylation sites, where BRF1 activity could be regulated. Serine 92 (S92) was identified by coworkers as a protein kinase B (PKB/Akt) phosphorylation site. To confirm this finding *in vivo* a phospho-specific antibody was raised in rabbits. Using this antibody, phosphorylation of S92 could be corroborated *in vivo*. Further, it could be shown that ARE-containing mRNA is stabilized under conditions of phosphorylated BRF1. Inhibitor experiments indicated that S92 is not only phosphorylated by PKB, but also by at least one other kinase, probably from the ERK1/2 MAPK pathway. Apart from S92, also other sites seem to be phosphorylated, as arsenite-treatment of mouse fibroblasts (NIH3T3) or insulin-treatment of rat fibroblasts, which overexpress the human insulin receptor (HIRc-B), result in a protein phosphatase sensitive shift of BRF1 on SDS-PAGE. These additional phosphorylation events were analyzed in a second part. Comparison of the sequence of all three Tis11 protein family members revealed that S203 in BRF1 is homologous to the MK2 target site S178 in TTP. Indeed, S203 seems to be phosphorylated. Inhibitor experiments suggested that the ERK pathway might be involved. In addition, mutation of S203 renders BRF1 insensitive to PKB and

MK2 mediated inhibition in cotransfection ActD-chase experiments and, therefore, seems to play a role in regulation of BRF1 activity.
Another way to control the activity of AUBPs is the regulation of their localization in the cell. Therefore, in a third part, the question was addressed, where the AUBPs HuR, $AUF1_{p37}$, BRF1 and TTP are localized in the cell and whether their localization might play a role in regulating the activity of these proteins. The effects of activated signaling cascades, known to stabilize ARE-mRNA, on AUBP localization were investigated. In the case of p38 MAPK and PI3-K stabilization of ARE-mRNA is, indeed, accompanied by translocation of the stabilizing AUBPs HuR and $AUF1_{p37}$ to the cytoplasm. Surprisingly, PKB, a downstream kinase of PI3-K, does not affect HuR and $AUF1_{p37}$ localization, indicating that PI3-K regulates their localization via another pathway. Probably, the effect is mediated via PKC, as stimulation by TPA, a potent activator of PKC, does also lead to cytoplasmic accumulation of HuR and $AUF1_{p37}$. In addition, HuR localization alters early during mitosis. At the onset of prophase, HuR is exported to the cytoplasm, indicating, that HuR might play a role in mitosis.
In the case of BRF1 and TTP no changes in localization could be observed in response to external stimuli. These two proteins are equally distributed in the cell, indicating that nuclear storage of these proteins cannot account for their inactivation.
To shed light on the physiological role of BRF1, a cell line was constructed with doxycycline-repressible BRF1 expression. This line was used in the last part of this thesis, to investigate, whether BRF1 plays a role in cell cycle control. Overexpression of BRF1 did accelerate progression of serum starved mouse fibroblast cells from G0/G1 phase to S phase. Downregulation of BRF1 by siRNA, on the other hand, did have the opposite effect. However, the effect was reproducible but only marginal. Therefore, the project was discontinued at this stage.
Taken together, the data presented gives insight into the mechanisms, linking extracellular signaling to mRNA stability. For BRF1 two sites of regulation, namely S92 and S203 could be identified. Further, export of the two stabilizing AUBPs HuR and $AUF1_{p37}$ seems to play an important role in ARE-mRNA stability control. A dual role could be assigned to the PI3-K signaling pathway: PI3-K inactivates the ARE-mRNA destabilizing protein BRF1 by PKB mediated phosphorylation at S92 and, via a PKB independent pathway, PI3-K exports HuR from the nucleus, further stabilizing ARE-containing transcripts.

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Introduction

Posttranscriptional Regulation of Gene Expression by Degradation of Messenger RNA

The flow of genetic information from DNA to protein is highly regulated at multiple stages. In addition to the well-studied control at the level of transcription and translation, the turnover of messenger RNA (mRNA) plays a crucial role in determining the amount of a given protein to be synthesized. The sum of mRNA synthesis and degradation affects both, the amount of protein that can be synthesized with a given translation rate and also the duration of translation. By transiently stabilizing short lived transcripts, steady state mRNA levels can be efficiently elevated within precisely controlled time windows, without changing the rate of transcription (Ross, 1995). This mechanism is used in many cases to quickly adapt the cells to stress signals, as for example in macrophages stimulated by IL-1 where increased cytokine production occurs (Huang *et al.*, 2000; Tebo *et al.*, 2000). Figure 1 shows, how mRNA levels change, if transcription is considered independent of the RNA concentration and degradation follows first order kinetics (dependent on the concentration of the substrate) (Hargrove *et al.*, 1989): With a constant transcription rate high levels of slow decaying mRNA accumulate. These levels remain high for a long time after transcription is turned off (panel A). If the mRNA is rapidly turned over, less message can accumulate, but the steady state is reached more quickly (compare panel A and B). Upon transcription stop, mRNA with a rapid decay disappears very quickly. By changing the stability of mRNA, high levels of mRNA can be produced that are degraded rapidly (panel C).

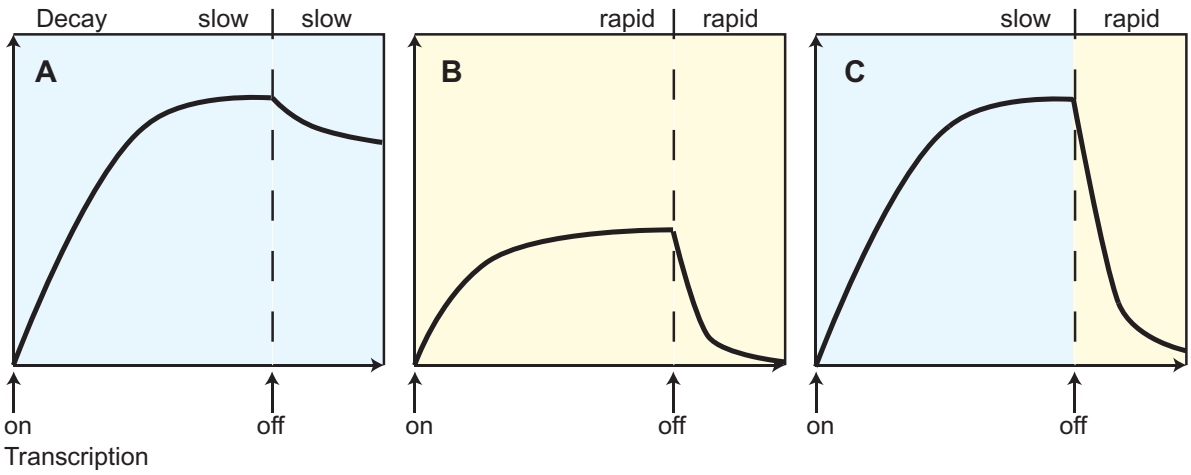


Figure 1: Levels of slowly (**A**) or rapidly decaying mRNA (**B**) in arbitrary units plotted against time. At the dotted line transcription is turned off and in panel **C**, stable mRNA is destabilized.

mRNA Degradation

When messenger RNA was discovered more than 40 years ago in *E.coli*, one of its main properties was instability, as seen by pulse labeling (Brenner *et al.*, 1961; Gros *et al.*, 1961). Half-lives of mRNAs range from 30 seconds to 20 minutes in *E. coli*. In eukaryotes mRNA turnover is slower, but half-lives are still usually shorter than generation time ranging from 20 minutes to over 24h. To assure proper function of the newly transcribed RNA, it is routed through many mRNA-protein complexes that mediate splicing, polyadenylation, capping, nuclear export, translation and finally degradation. Aberrant RNA needs to be detected and degraded. These processes have to be controlled tightly in a way that every RNA meets its proper fate. How does a cell distinguish between RNA that has to be processed and RNA that has to be degraded completely? In the last few years, substantial progress has been made in understanding, how these processes work and which enzymes are involved. Interestingly, it has been found that the same or at least similar enzyme-complexes are responsible for RNA processing, maturation and degradation.

In Prokaryotes

In bacteria, mRNA degradation appears to be initiated by one or more endonucleolytic cleavages. This step is followed by 3'-5' exonucleolytic digestion of the resulting mRNA fragments. Three endoribonucleases and three 3'-5' exoribonucleases have been identified in bacteria. The major endonucleolytic enzyme in mRNA decay is RNase E (Rne). Apart from its function in mRNA degradation it also plays a role in processing the 9S ribosomal RNA (rRNA) to the 5S form. RNase E cleaves single stranded mRNA at specific sites typically in an AU-rich context. Interestingly, a free 5'-end enhances the activity of RNase E (Bouvet and Belasco, 1992), indicating that determinants at the 5'-end of mRNAs regulate its susceptibility to endonucleolytic cleavage. As cleavage by RNase E again produces free 5' monophosphates, further cleavage of the remaining fragments is accelerated by the new free 5'-ends and degradation, therefore, seems to processes in a 5'-3' direction. Another ribonuclease with overlapping specificity to RNase E is RNase G. The third endonuclease, RNase III, cleaves double strand regions of mRNA.

The exonucleases responsible for RNA decay include the hydrolytic RNase II and polynucleotide phosphorylase (PNPase), a phosphorolytic enzyme from the RNase PH family of exonucleases and oligoribonucleases, which is required for the breakdown of small 2-5 nucleotide fragments. Secondary structures on mRNA impede proper RNA degradation by RNase II and PNPase. However, the discovery of a multiprotein-complex termed the degradosome during purification of RNase E (Ehretsmann *et al.*, 1992; Carpousis *et al.*, 1994; Py *et al.*, 1994; Carpousis *et al.*, 2001) provided a satisfactory solution to this problem. The key mRNA decay RNases in *E.coli*, RNase E and PNPase, are associated together with the DEAD-box containing ATP-dependent helicase RhIB in this complex (Py *et al.*, 1996), which is able to unwind stable secondary structures, making the RNA accessible to the combined action of endo- and exoribonucleases. Other components are also found in the degradosome, for which no function has been assigned yet. Apart from RNA decay, the degradosome is also involved in processing 9S to 5S rRNA.

An additional important enzyme in mRNA degradation is the poly(A)polymerase PAP. It has been found to polyadenylate prokaryotic RNA at their 3'-end. Poly(A) tails typically range from 10 - 40 nucleotides and have been found to decrease the half-lives of mRNAs. This has been explained

by a model, where extension of a RNA 3'-end by the addition of a poly(A) tail facilitates binding of RNase II and PNPase, which is inhibited by double stranded secondary structures, thereby enhancing exonucleolytic degradation. Further details of prokaryotic mRNA turnover are reviewed in (Steege, 2000; Carpousis, 2002).

In Eukaryotes

In yeast mRNA decay is initiated by removal of the poly(A)-tail. Two distinct deadenylation activities were found: The poly(A)-nuclease (Pan2/3) complex trims nascent poly(A)-tails in the nucleus and the cytoplasm and Ccr4 (Carbon catabolite repression 4) / Caf1 (Ccr4 associated factor 1), which have previously been defined as transcriptional regulators, are required for optimal deadenylation. The dual role of Ccr4/Caf1 in deadenylation and transcription points to a coupled regulation of these two processes.
After deadenylation the 5' cap of mRNA is removed. Several factors have been identified, which are involved in this process, including Dcp1/2, Vps16, Pat1 and the Lsm proteins. After decapping, the mRNA body is degraded by the 5' exonuclease Xrn1 (for review see (Wilusz *et al.*, 2001)). Figure 2 shows an overview of the mRNA degradation pathways present in eukaryotes.

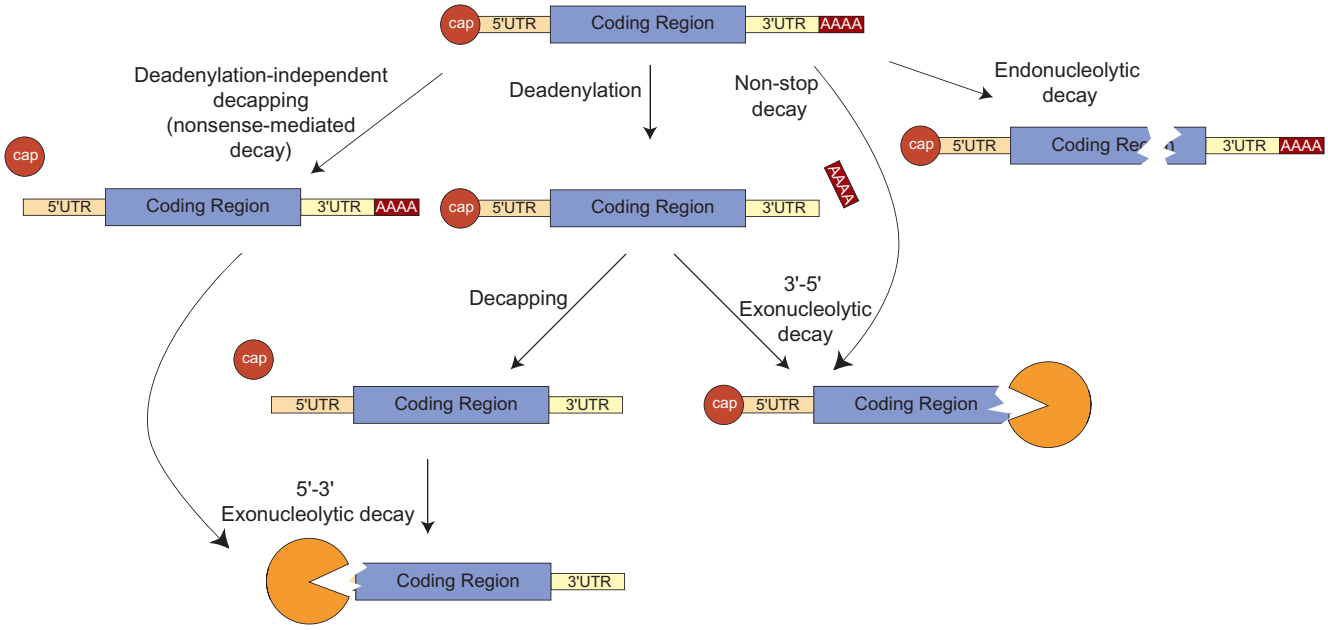


Figure 2: Summary of eukaryotic mRNA turnover. Adapted from (Parker and Song, 2004).

Although decay in yeast is mainly directed 5'-3', there is 3'-5' exonuclease activity present. Similar to the proteasome responsible for the degradation of proteins and the degradosome in bacteria described in the previous chapter, a multiprotein complex named the exosome was found to exert this function (Mitchell *et al.*, 1996; Mitchell *et al.*, 1997; Allmang *et al.*, 1999b). The exosome contains 3'-5' exonucleases. It has been found to trim 5,8S rRNA (Mitchell *et al.*, 1996), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) (Allmang *et al.*, 1999a) and to degrade

mRNA in an ATP-dependent manner from the 3'-end (Anderson and Parker, 1998). In yeast, the exosome consists of at least 11 different proteins, 10 of which are 3'-5' exonucleases (table 1). Six proteins of the complex are 3'-5' phosphorolytic enzymes related to the *E.coli* RNase PH (Rrp41p (for ribosomal RNA processing 41 protein), 42p, 43p, 45p, 46p and Mtr3p), Rrp44 is a hydrolytic enzyme related to RNase II in *E.coli* and the other components (Rrp4p, 40p and Csl4p) have a S1 RNA-binding domain. The fact that all these exosomal components are essential for viability (Mitchell *et al.*, 1997) indicates that, in addition to their ribonucleolytic activity, they are also crucial for correct assembly of the complex. The exosome is present in the cytoplasm and the nucleus, where an additional 3'-5' exoribonuclease (Rrp6p) forms part of the complex (Mitchell *et al.*, 1997; Allmang *et al.*, 1999b; Zanchin and Goldfarb, 1999). Although the structure of the exosome has not been resolved yet, based on homology of many exosomal components to the bacterial RNase PH, a ring like structure seems plausible, where the different nucleases surround the RNA (Symmons *et al.*, 2002). For efficient exosome function, associated factors are required. Ski2p and Mtr4p are helicases that disrupt secondary RNA structures and protein-RNA interactions. In addition, they seem to deliver the RNA to the exosome. The interaction of these proteins with the exosome may be direct or through binding of Ski3p and Ski8p, two proteins required for mRNA degradation (Anderson and Parker, 1998).

	Yeast	Human	*E.coli* homologues	Comments
Exosomal components	Rrp4p	hRrp4p	S1 RNA BD	hRrp4p is a functional homologue of the yeast component
	Rrp40p/Ski6p	hRrp40p	S1 RNA BD	homologues to Rrp4p
	Rrp41p	hRrp41p	RNase PH	hRrp41p is a functional homologue of the yeast component
	Rrp42p	hRrp42p	RNase PH	
	Rrp43p	-	RNase PH	
	Rrp44p/Dis3p	hDis3p	RNase II	hDis3p is a functional homologue of the yeast component
	Rrp45p	PM-Scl75 OIP2p	RNase PH	
	Rrp46p	hRrp46p	RNase PH	
	Mtr3p	-	RNase PH	
	Ski4p/Csl4p	hCsl4p	S1 RNA BD	hCsl4p is a functional homologue of the yeast component
	Rrp6p	PM-Scl100	RNase D	Interaction witn hRrp4p; only present in the nuclear complex
Associated proteins	Ski2p			RNA helicase
	Ski3p			specificity?
	Ski8p			specificity?
	Mtr4p			RNA helicase

Table 1: The table shows the exosomal components in yeast and human and their homologs in *E. coli*.

Similar to yeast, deadenylation is also the first step of the major mRNA decay pathway in mammalian cells. The poly(A)-ribonuclease (PARN/DAN) is the poly(A)-specific deadenylating nuclease in mammalian cells and *X. leavis* oocytes (Korner and Wahle, 1997; Korner *et al.*, 1998). The subsequent steps of mRNA decay in mammalian cells are poorly defined. Decapping may occur after deadenylation, followed by 3'-5' exonucleolytic digestion of the mRNA body. 5' exonucleases have also been identified in mammalian cells, suggesting bidirectional degradation, however, *in vivo* 3'-5' degradation was found to be the major pathway (Mukherjee *et al.*, 2002). In humans, an exosome complex has been purified as well. The human homolog of Rrp4p (hRrp4p) was found to be in a complex in HeLa cells, comparable to the yeast exosome (Mitchell *et al.*, 1997). hRrp4p associates

with PM-Scl100 and PM-Scl75, which are components of the PM-Scl particles (Allmang *et al.*, 1999b), recognized by the autoimmune sera of patients suffering from Polymyositis-scleroderma overlap syndrome. The PM-Scl particles turned out to be the human counterpart of the yeast exosome. It contains 11-16 proteins, 10 of which are homologs to the yeast exosome subunits. The human exosome is located in the cytoplasm and the nucleus, with one component (PM-Scl100) only present in the nuclear complex. Four of the human proteins (hRrp4p, 41p, 44p and Csl4) have been shown to suppress the growth defect of mutant yeast strains, lacking the functional yeast homolog (Mitchell *et al.*, 1997; Allmang *et al.*, 1999b). In addition to the exoribonucleases, helicases are also associated with the mammalian exosome (Tran *et al.*, 2004).

For some mammalian mRNAs, (e.g. IGF-II, transferrin receptor, *c-myc*) endonucleolytic, deadenylation-independent cleavage has been reported (Beelman and Parker, 1995; Lemm and Ross, 2002) (See also Fig. 2). The resulting fragments are then further degraded in the 3'-5' and the 5'-3' direction.

Comparing prokaryotic mRNA decay to the eukaryotic process, several striking features emerge: First, in eukaryotic cells the 5'-3' decay pathway can be found in addition to the evolutionary conserved 3'-5' degradation pathway. Second, the poly(A) tails in prokaryotes and eukaryotes have an opposite function. Whereas in eukaryotes the poly(A) tail protects mRNA from degradation, in prokaryotes it destabilizes the RNA. Interestingly in both, prokaryotes and eukaryotes, a multiprotein complex, consisting mainly of exoribonucleases, is responsible for the 3'-5' degradation of mRNA. Why these enzyme-complexes are formed is not clear yet. Probably, the high concentration of ribonucleases and helicases assures higher efficiency of mRNA degradation. Maybe several different ribonucleases perform distinct steps in processing or degrading mRNA, reducing the amount of free premature end products.

Another mechanism of mRNA degradation was discovered in 1979. It was found that nonsense mutations in eukaryotic mRNA creating a premature termination codon (PTC) rendered the message unstable due to the so-called nonsense-mediated decay (NMD) (Chang *et al.*, 1979; Losson and Lacroute, 1979). After splicing, proteins are deposited at the exon-exon junctions, known as the exon junction complex (EJC). It is thought that, in mammals, these factors are not only required for proper mRNA splicing and export, but serve also as markers. These markers are removed by the ribosome during the first round of translation. As introns are normally only present in the coding region, any termination codon placed upstream of an EJC is defined as premature and is recognized by the "surveillance complex" containing the Upf proteins. Subsequent degradation is thought to occur via deadenylation-independent decapping followed by 5'-3' exonucleolytic degradation (see Fig. 2). In the fruit fly the EJC is dispensable for NMD, indicating that PTC-recognition occurs independently of the exon boundaries (Gatfield *et al.*, 2003). Further, Gatfield showed that in drosophila NMD is initiated by endonucleolytic cleavage (Gatfield and Izaurralde, 2004). NMD reduces the amount of putatively harmful, aberrant proteins to be synthesized. Interestingly, inactivation of the NMD pathway has also been shown to affect levels of a variety of "normal" (none PTC-containing) mRNA, indicating that not only aberrant mRNA decays via this pathway (reviewed in (McKendrick, 2003)).

Recently, a process named nonstop decay was discovered, where the absence of a stop codon is detected by the cell and leads to targeted degradation of the aberrant message (Frischmeyer *et al.*, 2002). The degradation of the mRNA lacking a stop codon requires the exosome complex (van Hoof

et al., 2002) and two additional associated factors, namely the Ski complex (consisting of Ski2p, Ski3p and Ski8p) and Ski7p. It is thought that the ribosome translates through the poly(A) tail and stalls at the 3'-end. Ski7p binds to the empty A-site of the ribosome and recruits the Ski complex together with the exosome to degrade the mRNA.

To defend an organism against exogenous (viruses) or endogenous (transposons) mobile genetic elements and as a mechanism to regulate gene expression, cells have developed an additional RNA degrading pathway. The process called RNA interference (RNAi) was first discovered in plants and subsequently found to be present in *C. elegans, D. melanogaster* and mammals. Double stranded RNA (dsRNA) is processed into small 21-25nt RNA fragments by Dicer, an evolutionary conserved RNase III family member. The small inhibitory RNAs (siRNAs) fragments guide a nuclease containing protein complex named RISC (for RNAi-induced silencing complex) to homologous substrate mRNA through base-pairing of the antisense strand of the siRNA. Subsequently, the targeted mRNA is degraded. (See reviews (Tijsterman *et al.*, 2002; Denli and Hannon, 2003) and references therein for more detailed information). In recent years RNAi has become a powerful tool in research to transiently knock down gene expression. Hairpin RNAs expressed from inducible promoters (e.g. tet-controlled promoter) are also processed into siRNA by Dicer and allow spatio-temporal controlled RNAi (Elbashir *et al.*, 2001; Brummelkamp *et al.*, 2002).

cis-Determinants of mRNA Stability

Many short-lived transcripts carry *cis*-elements that regulate their stability probably by controlling the access of the mRNA to the decay machinery. These elements are found all over the RNA as shown in Figure 3.

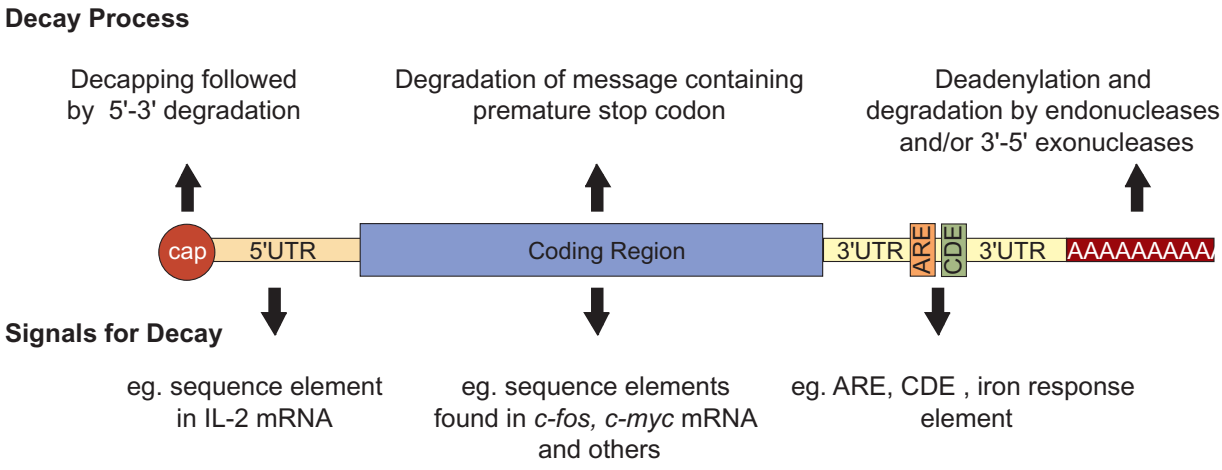


Figure 3: Schematic representation of an mRNA, its *cis*-elements and their function. Adapted from (Brennan and Steitz, 2001).

AU-rich Element

A major *cis*-element is the so-called AU-rich element (ARE), which is located in the 3' untranslated region (3'UTR) of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Shaw and Kamen, 1986; Chen and Shyu, 1995). A human database by Bakheet and coworkers revealed that 8% of all mRNAs, encoding a diverse functional repertoire of proteins, contain AREs (Bakheet *et al.*, 2001/ http://rc.kfshrc.edu.sa/ared). A recent statistical analysis by An *et al.* reported that AREs derive from the poly(T) regions complementary to Alu-repeats (An *et al.*, 2004). A general feature of these elements is a variable number of, often overlapping, AUUUA motifs, frequently placed in an U-rich context (class I and II according to Chen *et al.* (Chen and Shyu, 1995)). Another class of AREs (class III) (Peng *et al.*, 1996) lacks the AUUUA pentamer but is characterized by a U-rich region. However, in recent years this classification has declined in importance, as it turned out that the structural differences of these three classes did not necessarily translate into functional differences. KSRP, for instance, promotes decay of mRNAs containing AREs of either of the three classes (Gherzi *et al.*, 2004). In addition to the AUUUA motif, auxiliary domains seem to be important for the destabilizing activity of AREs (Winzen *et al.*, 2004). The ARE functions by targeting mRNA for rapid degradation. The functionality of this element was discovered by Shaw and Kamen, who introduced the ARE of GM-CSF into an α-globin reporter transcript, thereby destabilizing this message (Shaw and Kamen, 1986). Similar action has been reported for numerous AREs by mutational analysis e.g. for *c-myc* (Jones and Cole, 1987), *c-fos* (Shyu *et al.*, 1989), *c-jun* (Peng *et al.*, 1996), IL-3 (Hirsch *et al.*, 1995) or tumor necrosis factor α (TNFα) (Xu *et al.*, 1997).

AREs promote decapping (Gao *et al.*, 2001) and deadenylation followed by degradation of the mRNA body (Shyu *et al.*, 1991; Xu *et al.*, 1997) via the exosome (Chen *et al.*, 2001; Mukherjee *et al.*, 2002). Stabilization of short-lived ARE-containing transcripts by exogenous signals leads to a rapid accumulation of mRNA with a consequent increase of protein levels. Physiological examples are found in T-cells activated by immune stimulation (Lindsten *et al.*, 1989), mast cells responding to IgE-linked allergens (Wodnar-Filipowicz *et al.*, 1989; Wodnar-Filipowicz and Moroni, 1990), or macrophages stimulated by IL-1/TNFα (Huang *et al.*, 2000), where increased cytokine production occurs. Deregulated ARE-dependent mRNA turnover can contribute to oncogenic transformation (Schuler and Cole, 1988; Nair *et al.*, 1994; Stoecklin *et al.*, 2003a), inflammation (Carballo *et al.*, 1998) and immunopathology (Kontoyiannis *et al.*, 1999), underlining the physiological relevance of this process. In addition to mRNA turnover, the ARE has been reported to regulate translation of TNFα mRNA (Kontoyiannis *et al.*, 1999; Piecyk *et al.*, 2000). As discussed below, the ARE is recognized by AU-binding proteins, which seem to be the key players, regulating the stability of ARE-containing message.

Iron Responsive Element (IRE)

This stem loop structure (23-27 bases in length) is present in the 3'UTR of transferrin receptor (TfR) mRNA and in the 5'UTR of ferritin mRNA. The transferrin receptor imports iron into cells and ferritin is a major intracellular iron-storage protein. At low iron levels, two iron regulatory proteins (IRP1/2) bind to the IRE of TfR mRNA, thereby stabilizing this transcript and allowing more iron uptake. Binding of IRP1/2 to the 5' UTR of ferritin mRNA, on the other hand, blocks translation and prevents iron sequestration (reviewed in (Rouault and Klausner, 1997))

C-rich Elements

A C-rich stretch is found in the α-globin transcript. It was shown to be bound by the so-called α-complex consisting of α-CP1, α-CP2 and AUF1 (Wang *et al.*, 1995; Kiledjian *et al.*, 1997). The α-CP proteins interact with the poly(A) binding protein (PABP), enhancing its binding efficiency to the 3'UTR of α-globin. This RNA dependent interaction stabilizes mRNA by protecting it from deadenylation-dependent degradation (Wang *et al.*, 1999; Wang and Kiledjian, 2000).

Constitutive Decay Element (CDE)

Another decay promoting element in the 3'UTR of TNFα has been characterized in our group (Stoecklin *et al.*, 2003b). The CDE mediates decay of the message when transferred into an β-globin reporter construct. This decay is, in contrast to the ARE-mediated decay, not abrogated by lipopolysaccharide (LPS)-treatment in RAW 264.7 cells or by activation of p38 MAPK or PI3-K in NIH3T3 cells; therefore the element was named constitutive decay element. So far, no consensus motif has been characterized for the CDE and it is not known whether a similar element is present in other transcripts. Recent work in our lab (Bernd Rattenbacher, unpublished data) showed, that nucleolin interacts with the CDE and may be involved in destabilizing the TNFα message.

Histone 3' Terminal Stem Loop

Histone mRNAs lack a poly(A)-tail but have a 3' terminal stem loop motif (6bp stem and a 4bp loop) (Pandey and Marzluff, 1987), which mediates the cell cycle dependent decay via a stem loop binding protein (Whitfield *et al.*, 2000). Interestingly, histone proteins promote degradation of its own mRNA in an *in vitro* decay assay, but not of other mRNA, such as *c-myc* or γ-globin (Peltz and Ross, 1987).

Insulin-Like Growth Factor II (IGF-II) Stem Loop

Human IGF-II mRNA is subjected to site-specific endonucleolytic cleavage in the 3' untranslated region. This is the first and rate-limiting step in degradation of this message. Scheper *et al.* have identified a conserved stable double-stranded RNA stem structure that is essential for cleavage (Scheper *et al.*, 1995). Not only the double-stranded character but also the sequence of the stem is important for efficient cleavage. Although van Dijk and coworkers have identified a protein interacting with this structure (van Dijk *et al.*, 1998), no follow-up publications confirmed its role in cleavage of IGF-II mRNA and the mechanism of this cleavage is still unknown.

Coding Region

mRNAs from *c-fos, c-myc* and β-tubulin contain instability elements within their coding region. The mCRD (for major coding region determinant) in *c-fos* is a purine-rich 320-nucleotide motif that renders the mRNA unstable. It encodes for a leucine zipper, crucial for *c-fos* protein function. A complex of proteins, including AUF1 isoform p37, poly(A)-binding protein (PABP), PABP-interacting protein (PAIP) and Unr, a purine-rich, RNA binding protein, binds to the mCRD (Grosset

et al., 2000). These findings implicate an interaction between the poly(A)-tail and the mCRD. However, the mechanism by which mCRD mediates degradation is not known yet.
β-tubulin mRNA stability is affected by the first 13 translated nucleotides. mRNA turnover in this case is translation dependent, as the first four translated amino acids display the recognition sequence conferring instability to the ribosome-bound mRNA (Gay *et al.*, 1987; Yen *et al.*, 1988).
Coding sequences in exon 2 and 3 of *c-myc* are important for downregulation of the transcript during myoblast differentiation (Yeilding and Lee, 1997). A protein specifically binding to these sequences and protecting *c-myc* mRNA from endonucleolytic cleavage has been identified (CRD-BP for CRD binding protein) (Prokipcak *et al.*, 1994). Evidence exists that translational pausing generates a ribosome-deficient region downstream of the pause site, and this region is exposed to endonuclease attack unless it is shielded by the CRD-BP (Lemm and Ross, 2002).

Location of Determinant on mRNA	Decay Determinant	Binding Proteins	Function*	mRNA	Comments
3'UTR	Iron-responsive element	IRP	d/s	Tfr	also translational regulator in ferritin
	C-rich element	α-CP1/2	s	α-globin	
		AUF1	s		
	CDE	Nucleolin	d?	TNF- α	
	Histone stem loop	Histone specific stem loop binding protein			
	IGF-II stem loop				Endonucleolytic cleavage site
Coding Region	mCRD	AUF1	d	*c-fos*	
		PABP	s?		
		PAIP	?		
		Unr	?		
	other CRDs			β-tubulin	
				c-myc	
5'UTR	JNK-response element	YB-1	s	IL-2	
		Nucleolin	s		
		IL1-a	s	Chemokine KC	

* s:stabilization / d:destabilization / e:enzymatic activity

Table 2: The different *cis*-determinants of mRNA stability and their binding proteins are summarized (without the ARE; see table 3).

5' Untranslated Region (5' UTR)

The 5' UTR cannot only regulate translation rate of a given transcript, but also the stability in a translation-dependent or independent manner. Studies by Chen *et al.* (Chen *et al.*, 2000) and Tebo *et al.* (Tebo *et al.*, 2000) have identified stabilizing elements in the 5'UTR of IL-2 and the chemokine KC mRNA. In the case of IL-2, a JNK-response element (JRE) stabilizes IL-2 message upon T-cell activation. YB-1 and nucleolin bind specifically to this element and seem to exert this effect.

AU-rich Element Binding Proteins (AUBPs)

Over the past few years several ARE-binding proteins (AUBPs) affecting mRNA turnover have been identified. Among these proteins we find stabilizing (HuR) or destabilizing (BRF1, BRF2, TTP, KSRP) proteins. None of those proteins have been shown to be enzymatically active. They rather seem to protect the RNA from the decay machinery (stabilizing factors) or to target the RNA for degradation. A summary of the currently known AUBPs is shown in table 3.

Protein	Localization*	Interacting Factors	Function**	Binding mRNAs	Comments
AUF1/hnRNP D	n / c	Hsp70	d/s	*c-myc, c-fos,* GM-CSF	4 isoforms; RRM binding motif
AUBF	c		?	*c-fos,* interferon, IL-3, GM-CSF	
AU-A, AU-B, AU-C	n (AU-A) / c		d	TNF, GM-CSF, *c-myc*	
AU-H			e		
hnRNP A1	n		d	GM-CSF, IL-2, *c-myc*	RRM binding motif
hnRNP C	n		d		
HuR	n	SETα/β, pp32, APRIL	s	*c-myc, c-fos,* GM-CSF, TNF-α	RRM binding motif
HuD, Hel-N1, HuC	n		s		
TTP	n / c	14-3-3	d	TNF-α, GM-CSF, IL-3	CCCH zinc finger
BRF1	n / c	14-3-3	d	TNF-α, IL-3	CCCH zinc finger
BRF2	n / c		d	TNF-α	CCCH zinc finger
KSRP	n / c		d	*c-fos*	KH motif
GAPDH	n / c		e		
PM-Scl75	n / c	exosome	d	TNF-α, GM-CSF	exosome component

*n: nuclear / c:cytoplasmic
** s:stabilization / d:destabilization / e: enzymatic activity

See text for references

Table 3: AU-rich element Binding Proteins

HuR (HuA)

HuR was originally identified in *D. melanogaster* as an essential factor for neural development (Campos *et al.*, 1985). It is a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins (Good, 1995; Ma *et al.*, 1996). By gel shift (Myer *et al.*, 1997) and UV-crosslinking experiments (Fan *et al.*, 1997), HuR has been shown to bind to a variety of ARE-mRNA, including the ones from IL-3 and *c-fos* (Ma *et al.*, 1996). Overexpression (Peng *et al.*, 1998; Fan and Steitz, 1998a; Ming *et al.*, 2001; Chen *et al.*, 2002), antisense RNA (Wang *et al.*, 2000a; Wang *et al.*, 2000b) and RNAi (Raineri *et al.*, 2004) experiments later suggested a role for HuR in stabilizing ARE-containing mRNA, possibly by binding to AREs (mainly class I and II) and protecting the message from degradation.

HuR, as well as the other Hu-family proteins, contains three classical RNA recognition motifs (RRMs). Deletion of the third of these RRMs, which has been suggested to bind the poly(A)-tail (Ma *et al.*, 1997), abolishes HuRs ability to stabilize ARE-containing mRNA (Fan and Steitz,

1998a). Between RRM2 and 3 there is a nucleo-cytoplasmic shuttling sequence (HNS for HuR nucleo-cytoplasmic shuttling sequence) (Fan and Steitz, 1998b) similar to the M9 domain in hnRNP A1. Increased cytoplasmic localization of the predominantly nuclear HuR correlates with stabilization of ARE-containing mRNA: e.g. stabilization of p21 (Wang *et al.*, 2000b) and RhoB message (Westmark *et al.*, 2004) upon UV irradiation or cyclin A and B1 stabilization in late G1/S and G2 phase of the cell cycle (Wang *et al.*, 2000a) is accompanied by accumulation of HuR in the cytoplasm. Also heat shock, a stress known to stabilize ARE-mRNA (Laroia *et al.*, 1999), leads to increased cytoplasmic localization of HuR (Gallouzi *et al.*, 2000). It is thought that HuR escorts the mRNA out of the nucleus, thereby increasing its stability (Fan and Steitz, 1998a; Fan and Steitz, 1998b).

Four HuR-interacting proteins have been identified (Brennan *et al.*, 2000): SETα/β (von Lindern *et al.*, 1992; Matsumoto *et al.*, 1993;), pp32 (Malek *et al.*, 1990) and APRIL (Mencinger *et al.*, 1998). These proteins bind to the RRM3. Three of these binding partners (SETα/β and pp32) have previously been identified as phosphatase 2A (PP2A) inhibitors (Li *et al.*, 1996; Saito *et al.*, 1999), suggesting that PP2A is involved in regulating ARE-containing mRNA decay. pp32 and APRIL shuttle between the nucleus and cytoplasm, as shown by heterokaryon fusion experiments and interact with the nuclear export factor CRM1. These proteins, therefore, seem to be involved in modulating HuR export. In human colon cancer cells (Dixon *et al.*, 2001) and tumors of the central nervous system (Nabors *et al.*, 2001) HuR overexpression was detected, which could account for the increased stability of ARE-containing COX2, VEGF and IL-8 mRNA in these cells. Two independent reports claim a role of HuR in muscle differentiation. Overexpression of HuR in mouse embryonic muscle cells stabilizes two myogenesis-specific transcription factors (myogenin and MyoD) and drives differentiation of these cells (Figueroa et al., 2003), whereas RNAi against HuR has the opposite effect (van der Giessen et al., 2003).

AU-Binding Factor1 (AUF1/ hnRNP D)

AUF1 was first identified by Brewer and coworkers as a promoter of ARE-mRNA decay in K562 cells (Brewer, 1991). Although recombinant AUF1 lacked decay promoting activity in early experiments (Zhang *et al.*, 1993), later work showed that increased expression of AUF1 is associated with rapid decay of ARE-mRNA in peripheral blood mononuclear cells (PBMCs) (Buzby *et al.*, 1996; Buzby *et al.*, 1999), smooth muscle cells (Pende *et al.*, 1996) and monocytes (Sirenko *et al.*, 1997).

AUF1 exists as four different isoforms, arising from alternative splicing: A 37 kDa core protein (p37), p40 containing an additional N-terminal insertion (exon 2), p42 with a C-terminal insertion (exon 7) and p45, containing exon 2 and 7. The presence of exon 7 (in p42 and p45) shifts the nucleo-cytoplasmic distribution of the protein to the nucleus and it blocks ubiquitination, whereas the isoforms lacking exon 7 are targeted to the ubiquitin-proteasome degradation pathway. Degradation of $AUF1_{p37}$ and $AUF1_{p40}$ correlates with rapid decay of ARE-containing mRNA, indicating that these two isoforms are mRNA stabilizing components (Laroia *et al.*, 1999; Laroia *et al.*, 2002; Laroia and Schneider, 2002). The absence of exon 2 (p37, p42) has been associated with high affinity ARE-binding (DeMaria *et al.*, 1997). Forced expression of p37 and p42 antagonizes ARE-mRNA stabilization, accompanying hemin-induced erythroid differentiation of K562 cells (Loflin *et al.*, 1999). On the other hand, overexpression of all four isoforms in 3T3 cells stabilizes

ARE-message (Xu *et al.*, 2001). The precise role of AUF1 in ARE-mRNA turnover is still a matter of debate, however, the emerging models assign stabilizing and destabilizing roles to the different isoforms depending on the cell type, the cellular environment and the type of ARE (Xu *et al.*, 2001; Raineri *et al.*, 2004). AUF1 seems to regulate mRNA stability by changing the structure of the RNA in response to signaling pathways. $AUF1_{p40}$ has been reported to keep the bound mRNA in a condensed conformation, which changes upon phosphorylation of S83 and S87 (Wilson *et al.*, 2003a; Wilson *et al.*, 2003b).
In addition to the role of AUF1 in ARE-mRNA turnover, these proteins have also been shown to be part of the α-globin mRNA stability complex (Kiledjian *et al.*, 1997) and to play a role in the mRNA decay directed by the *c-fo*s mCRD (Grosset *et al.*, 2000). Furthermore, AUF1 is involved in telomere maintenance (Eversole and Maizels, 2000) and transcriptional activation (Fuentes-Panana *et al.*, 2000).

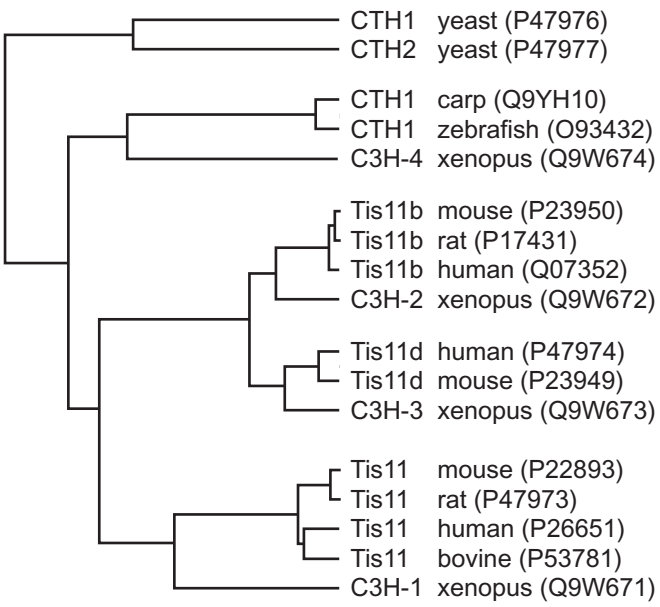
Tis11 Family

The Tis11 (Tetradecanoyl phorbol acetate (TPA)-induced sequence 11) family of proteins consists of 3 members in mammals: Tristetraprolin (TTP), Butyrate response factor 1 (BRF1) and 2 (BRF2), (encoded by Zinc finger protein 36 (Zfp36), Zfp36 like 1 (Zfp36L1) and Zfp36L2 respectively). A common feature of these proteins is the presence of two $C\text{-}X_8\text{-}C\text{-}X_5\text{-}C\text{-}X_3\text{-}H$ zinc finger motifs, each preceded by a conserved YKTEL sequence (Varnum *et al.*, 1991; Amann *et al.*, 2003; Michel *et al.*, 2003; Hudson *et al.*, 2004). All Tis11 family members bind to the ARE of TNFα (Lai *et al.*, 2000). Two homologs of this family were cloned in yeast (Thompson *et al.*, 1996) and in frog and fish a fourth family member has been found (De *et al.*, 1999).

Tristetraprolin (TTP/ Tis11/ Zfp36p/ NUP475/ GOS24)

TTP was found independently by three different groups: Herschman and colleagues identified a partial clone of the cDNA in a screen for phorbol-ester-responsive genes and named it Tis11 (Varnum *et al.*, 1989). DuBois *et al.* cloned TTP under the name of NUP475 (nuclear protein 475) as a serum-induced gene (DuBois *et al.*, 1990), and the Blackshear lab found it in a screen for genes that are rapidly turned on at the transcriptional level in response to insulin (Lai *et al.*, 1990). Due to the presence of three characteristic P-P-P-P-G motifs, Blackshear and coworkers named the protein Tristetraprolin (TTP). Apart from TPA, serum and insulin, TTP mRNA expression was later shown to be induced with similar kinetics by a variety of growth factors (EGF, FGF), by LPS and by TNFα (Carballo *et al.*, 1998; Fairhurst *et al.*, 2003). Several years after cloning, the function of TTP came apparent, when TTP knockout mice were generated (Taylor *et al.*, 1996). TTP (-/-) mice are born normal, but, within a few months, develop a phenotype characterized by loss of weight, severe polyarticular erosive arthritis and myeloid hyperplasia in and outside of the bone marrow. They also developed alopecia, dermatitis, conjunctivitis and autoimmunity. The observed phenotype resembled the one induced by chronic administration of TNFα (Keffer *et al.*, 1991; Ulich *et al.*, 1993). Injection of a monoclonal antibody against TNFα could suppress this phenotype (Taylor *et al.*, 1996), indicating that the phenotype arises due to TNFα overproduction. Indeed, macrophages from TTP (-/-) mice exhibit increased TNFα and GM-CSF mRNA stability (Lai *et al.*, 2000), which

A



B

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human Tis11     RYKTELCRTF SESGRCRYGA KCQFAHGLGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIH
mouse Tis11     RYKTELCRTY SESGRCRYGA KCQFAHGLGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIH
rat Tis11       RYKTELCRTY SESGRCRYGA KCQFAHGPGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIH
bovine Tis11    RYKTELCRTF SESGRCRYGA KCQFAHGLGE LRQASRHPKY KTELCHKFYL QGRCPYGSRC HFIH
xenopus C3H-1   RYKTELCRTF SETGTCKYGA KCQFAHGKIE LREPNRHPKY KTELCHKFYL YGECPYGSRC NFIH
human Tis11b    RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
mouse Tis11b    RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
rat Tis11b      RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
xenopus C3H-2   RYKTELCRPF EENGSCKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
human Tis11d    RYKTELCRPF EESGTCKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
mouse Tis11d    RYKTELCRPF EESGTCKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
xenopus C3H-3   RYKTELCRPF EENGACKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIH
carp CTH1       RYKTELCSRY AETGTCKYAE RCQFAHGLHD LHVPSRHPKY KTELCRTYHT AGYCVYGTRC LFVH
zebrafish CTH1  RYKTELCSRY AETGTCKYAE RCQFAHGLHD LHVPSRHPKY KTELCRTYHT AGYCVYGTRC LFVH
xenopus C3H-4   RYKTELCSRY AESGFCAYRN RCQFAHGLSE LRPPVQHPKY KTELCRSFHV LGTCNYGLRC LFIH
yeast CTH1      LYKTELCESF TIKGYCKYGN KCQFAHGLNE LKFKKKSNNY RTKPCINWSK LGYCPYGKRC CFKH
yeast CTH2      LYKTELCESF TLKGSCPYGS KCQFAHGLGE LKVKKSCKNF RTKPCVNWEK LGYCPYGRRC CFKH
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C

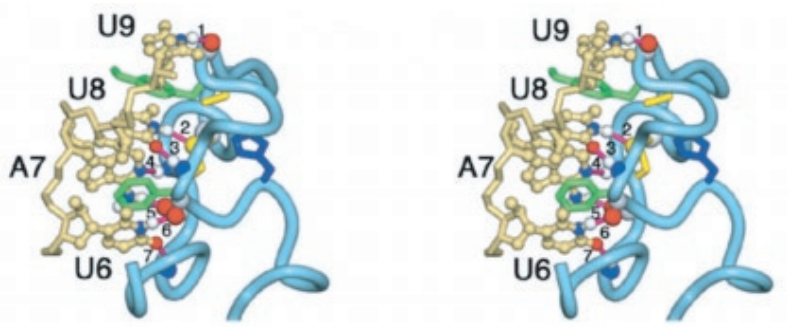


Fig. 4: **A:** Phylogenetic tree of the known Tis11 family members. **B:** Alignment of the zinc finger regions of the Tis11 protein family. **C:** Stereo view showing the structure of the BRF2 zinc finger bound to UUAUUUAUU (Hudson *et al.*, 2004)

could explain the TNFα overproduction in the knockout mice. Further investigations revealed that TTP can bind to the ARE of TNFα mRNA dependent on the two zinc fingers (Lai *et al.*, 1999), thereby destabilizing the message (Carballo *et al.*, 1998). The 77 amino acids surrounding the two zinc fingers are sufficient for deadenylation and decay induction (Nakielny and Dreyfuss, 1996). Recently, it has been shown independently by Brooks *et al*. and Tchen *et al*. (Brooks *et al.*, 2004; Tchen *et al.*, 2004) that TTP binds to its own 3' UTR and destabilizes its mRNA. This provides a negative feedback loop, controlling the levels of TTP. In our lab, TTP was found to reverse the mutant phenotype of the ARE-decay defective slowC cells lacking BRF1 expression (Stoecklin *et al.*, 2000). It was found that TTP promotes deadenylation of ARE-mRNA *in vitro* (Lai *et al.*, 2003) and *in vivo* (Lai *et al.*, 1999). Interestingly, deadenylation is not the only way, how TTP affects stability of ARE-mRNA, as the poly(A)-tail in GM-CSF, TNFα or IL-3 is not required for TTP mediated mRNA turnover (Lai and Blackshear, 2001). The fact that non-RNA-binding TTP inhibits degradation of ARE-mRNA suggests that TTP function requires interacting proteins (Lai *et al.*, 2002). Indeed, Chen and coworkers could show that TTP promotes degradation of ARE-mRNA by recruiting the exosomal decay machinery (Chen *et al.*, 2001).

TTP is a nucleo-cytoplasmic shuttling protein containing a nuclear localization signal (NLS) between amino acids 76-189 and a leucine-rich nuclear export signal (NES) in its N-terminus (Murata *et al.*, 2002; Phillips *et al.*, 2002). In yeast, overexpression of the two zinc finger protein family members (CTH1/2) or mammalian TTP causes delayed entry of cells into exponential growth, depending on the zinc fingers (Thompson *et al.*, 1996). In mammalian cells continuous TTP expression causes apoptotic cell death and sensitizes cells to TNFα-induced apoptosis (Johnson *et al.*, 2000; Johnson *et al.* 2002). In addition to its decay promoting activity, TTP was also found to exert a suppressive effect on the promoter of TNFα and IL-8, which is released by p38 activation (Zhu *et al.*, 2001).

Butyrate Response Factor 1 (BRF1/ Tis11b/ Zfp36L1p/ Berg36/ ERF1/ cMG1)

BRF1 was originally cloned as an epidermal growth factor (EGF)-inducible gene in rat intestinal epithelial cells (Gomperts *et al.*, 1990) and its human homolog was cloned four years later (Bustin *et al.*, 1994). The human gene was mapped to chromosome 14q22-24 (Maclean *et al.*, 1995). BRF1 expression is induced by insulin, insulin-like growth factor 1 (IGF-1) (Corps and Brown, 1995) and by adrenocorticotropin (ATCH) (Chinn *et al.*, 2002). BRF1 expression is suppressed by butyrate, in contrast to TTP, which is not affected (Maclean *et al.*, 1998). Interestingly, sodium butyrate, a fermentation product of dietary fiber, inhibits colorectal cancer cell proliferation by inducing growth arrest, differentiation and apoptosis. Unpublished work in our lab by Ines Raineri and co-workers showed that downregulation of BRF1 by siRNA in embryonic stem cells induces morphological changes similar to differentiation. It has also been found that high levels of BRF1 contribute to leukemogenesis mediated by the fusion transcription factor AML1-MTG8 (Shimada *et al.*, 2000). Reduction of BRF1 levels could, therefore, be of benefit for cancer patients. On the other hand, continuous expression of BRF1 causes apoptosis in 3T3 and HeLa cells, but has no effect on TNFα induced apoptosis (Johnson *et al.*, 2000). Its physiological function in proliferation and apoptosis is therefore still a matter of debate. Recently it was shown that a BRF1 knockout is lethal at embryonic day 11 due to a defect in chorioallantoic fusion (Stumpo *et al.*, 2004).

BRF1 exhibits CRM1 dependent nucleo-cytoplasmic shuttling (Phillips *et al.*, 2002). It contains a NES in its C-terminus. BRF1 was identified in our lab by a functional screen aimed at finding genes responsible for ARE-dependent decay (Stoecklin *et al.*, 2002). A human fibrosarcoma cell line with frameshift mutations in both BRF1 alleles, therefore lacking BRF1 expression, was cloned (slowC). The slow ARE-mRNA degradation phenotype of this line could be reverted by transfection of BRF1. Further, BRF1 has been shown in a cell free system to stimulate deadenylation similar to TTP (Lai *et al.*, 2003).

Butyrate Response Factor 2 (BRF2/ Tis11d/ Zfp36L2/ ERF2)

BRF2 was cloned as the third member of the Tis11 protein family (Varnum *et al.*, 1991). The human BRF2 gene was cloned by Nie *et al.*, who claimed that it differs from its mouse homolog by encoding an additional 97 amino acids at its C-terminal end (Nie *et al.*, 1995). This has later been shown to be wrong (De *et al.*, 1999). BRF2 binds to and destabilizes TNFα mRNA (Lai *et al.*, 2000). The NMR structure of the BRF2 zinc fingers bound to a single stranded RNA oligonucleotide UUAUUUAUU was recently resolved by Hudson *et al.* (Hudson *et al.*, 2004) and showed that each of the two similarly folded zinc fingers binds to separate UAUU sites (Figure 4C). As the zinc finger motive is highly conserved among the different Tis11 family members (Figure 4B) the same structural features are to be expected for TTP and BRF1. Recently, it was shown by Ramos and colleagues (Ramos *et al.*, 2004) that partial BRF2 knockout mice lacking the first exon of BRF2, but containing parts of the following intron, are viable. The only observed phenotype is infertility of the females. Fertilized embryos stop cell division at the two-cell state. Fertility could be restored by transplantation of wild-type ovaries.
Similar to BRF1, BRF2 also contains a NES in its C-terminus and shuttles between the nucleus and cytoplasm in a CRM1 dependent fashion (Phillips *et al.*, 2002).

KSRP (FBP2)

KSRP (K homology-type splicing regulatory protein) was originally identified as a component involved in neuronal-specific *c-src* splicing (Min *et al.*, 1997). It contains 4 copies of the RNA binding K homology (KH) domain. These domains are essential for the function of KH proteins in differentiation in flies, worms and mammals (Adinolfi *et al.*, 1999). KH proteins are important regulators of metabolic processes in prokaryotes and eukaryotes. KSRP is a ubiquitously expressed, shuttling protein and binds to TNFα and *c-fos* AREs. It has been shown to interact with the exosome and is thought to target ARE-mRNA for rapid degradation via this complex (Chen *et al.*, 2001; Gherzi *et al.*, 2004).

CUGBP2 (NAPOR2/ ETR-3/ BRUNOL3)

CUGBP2 (CUG-binding protein2) is a prototype RNA binding protein of the CELF (CUGBP-ETR-3-like factors) family. CELF proteins contain three RRMs, two of which are located as tandem repeats similar to HuR. However, apart from these motifs no homology can be found between CUGBP2 and HuR (Choi *et al.*, 1998). CUGBP2 is induced during apoptosis. It binds to CUG repeats (found in myotonic dystrophy (Lu *et al.*, 1999)) and to the ARE of Cyclooxygenase-2 (COX-2). CUGBP2

expression is induced upon radiation. After binding to COX-2 mRNA, CUGBP2 stabilizes the message but inhibits its translation (Mukhopadhyay *et al.*, 2003). In addition, CUGBP2 is involved in editing (Anant *et al.*, 2001), splicing, and development of the central nervous system (Ladd *et al.*, 2001).

TIA-1, TIAR

TIA-1 (T-cell intracellular antigen-1) and TIAR (TIA-1 related protein) are RRM-containing proteins that bind specifically to the ARE of TNFα. Macrophages from mice lacking TIA-1 (TIA-1 (-/-)) produce significantly more TNFα protein than wild-type controls. However the half-life of TNFα transcripts is similar in wild type and TIA-1 (-/-) macrophages, indicating that TIA-1 does not regulate transcript stability but silences translation. Consistent with its role in translation, the absence of TIA-1 significantly increases the proportion of TNFα transcripts that associate with polysomes (Gueydan *et al.*, 1999; Piecyk *et al.*, 2000). The translational inhibition of TIA-1 is specific for TNF-α, as GM-CSF and Interferon-α are not affected. The TIA proteins are nucleo-cytoplasmic shuttling proteins. In stressed cells they translocate from the nucleus into the cytoplasm, where they accumulate in subcellular sites called stress granules. In addition to the TIA proteins, eukaryotic initiation factors (eIF3, 4E, 4G), PABP, TTP, HuR and AUF1 are also found in these cytoplasmic structures, where translationally silenced mRNA is stored during stress (for review see (Kedersha and Anderson, 2002)).

PM-Scl75

A link between the exosome and ARE-mediated decay was provided by Mukherjee *et al.*, who found that the exosomal component PM-Scl75, which is needed for efficient 3'-5' degradation, interacts with AREs (Mukherjee *et al.*, 2002). This interaction could be responsible for targeting the exosome to ARE-containing short-lived message, facilitating its degradation.

Signal Transduction Pathways and their Involvement in ARE-mRNA Stability Control

A cell senses changing environmental conditions via a complex network of signaling cascades. The incoming stimuli are integrated into the appropriate response. Many pathways have been deciphered up to now and many have been found to regulate mRNA turnover. Stress stimuli, including UV-exposure (Gorospe *et al.*, 1998; Wang *et al.*, 2000b), heat shock (Laroia *et al.*, 1999) and hypoxia (Paulding and Czyzyk-Krzeska, 2000), can lead to stabilization of ARE-mRNA. AUBPs are obvious candidate targets for signaling pathways, regulating mRNA turnover. The signaling pathways relevant to this work and their effect on mRNA turnover are discussed in the following section.

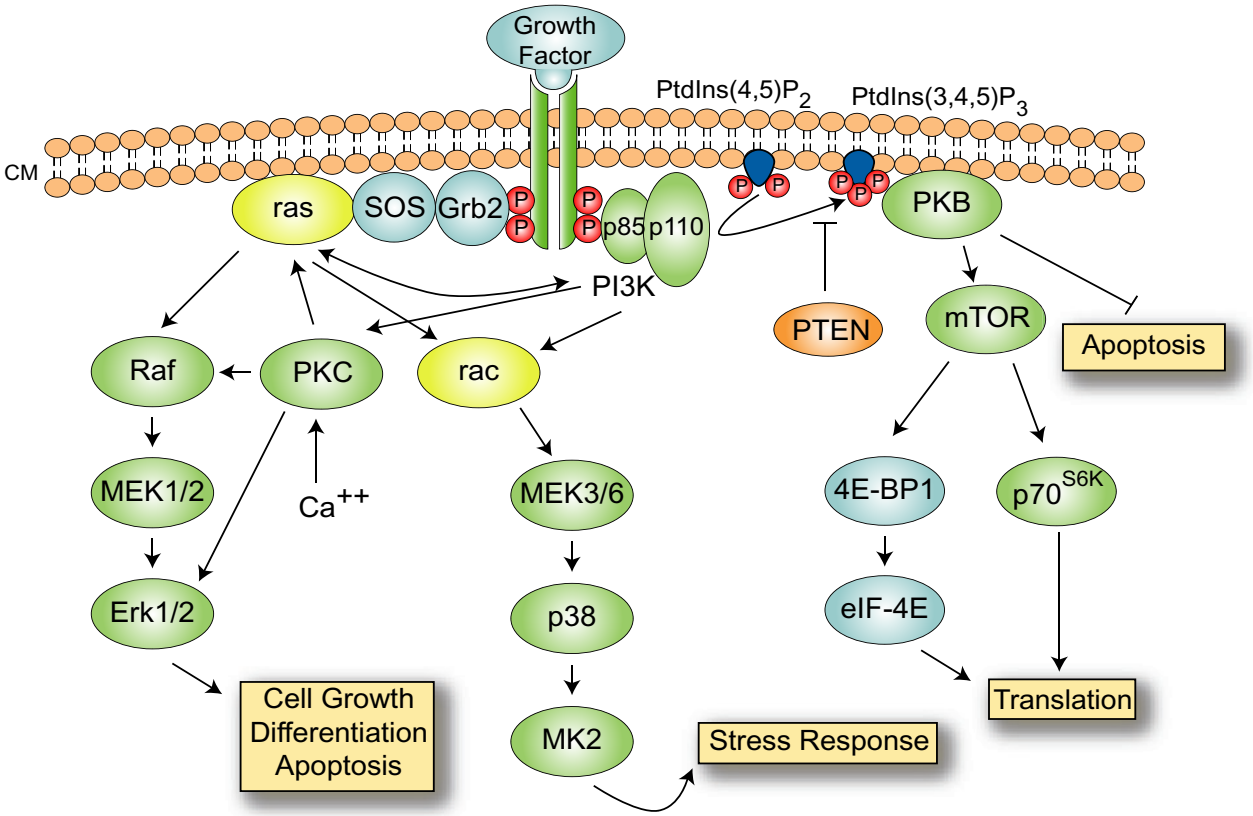


Fig. 5: Overview of the signal transduction pathways relevant to this work. Kinases are shown in green, small G-proteins in yellow, other factors in blue and the phosphate groups in red. Some of the most important biological responses of these pathways are mentioned. CM: Cell membrane.

Phosphatidylinositol 3-Kinase (PI3-K)

Activation of receptor tyrosine kinases (RTKs) or G protein coupled receptors (GPCRs) can result in the recruitment of PI3-K to the plasma membrane. This interaction can also be indirect, via intermediate phospho-proteins, such as insulin receptor substrates (IRS1 or 2). PI3-K consists of a regulatory domain (p85), which associates via its src homology 2 (SH2) domain with the RTK (or the IRS), and a catalytic domain (p110), which catalyses the transfer of phosphate to the D-3 position of the inositol ring of membrane localized phosphoinositides (PI). RTKs can also activate PI3-K indirectly via the small G-protein ras, which can bind the p110 subunit. The products of the reaction catalyzed by PI3-K (mainly phosphatidylinositol (3,4) bisphosphate (PtdIns(3,4)P_2) or PtdIns(3,4,5)P_3) act as signaling intermediates that regulate downstream signaling events. The phosphoinositide phosphatase PTEN hydrolyses PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 and, thereby, counteracts PI3-K signaling. Proteins containing pleckstrin homology (PH) domains are likely to bind to PtdIns(3,4,5)P_3. Several cytoplasmic kinases contain a PH domain. Among them the protein kinase B (PKB)/AKT is the best-characterized downstream effector of PI3-K. Another PH domain containing kinase is PDK1, which phosphorylates and activates PKB and PKC (reviewed in (Vivanco and Sawyers, 2002)).

In our lab Ming *et al.* (Ming *et al.*, 2001) have shown that transfection of an activated form of PI3-K stabilizes ARE-mRNA. This stabilization does not involve direct inactivation of TTP. Evidence is presented in this work and in (Schmidlin *et al.*, 2004) that PI3-K mediated stabilization acts via activation of PKB, which, at least partly, inactivates BRF1.

Protein Kinase B (PKB/AKT)

Since the cloning of PKB in the early nineties this protein kinase has emerged to play a central role in protein synthesis, transcription, cell growth, cell survival, angiogenesis and glycogen synthesis. It belongs to the AGC family of kinases together with the founding members PKA, PKG and PKC. PKB exists as three isoforms PKB α-γ (AKT1-3 respectively). It contains a N-terminal PH-domain, a central kinase domain and a C-terminal regulatory domain. Upon elevation of PtdIns(3,4,5)P_3 levels by PI3-K, PKB is recruited to the membrane via its PH-domain. This causes unfolding of PKB followed by phosphorylation at threonine 308 (T308) and serine 473 (S473) by phosphoinositide-dependent kinase 1 (PDK1) and a yet unidentified S473 kinase (S473K/PDK2). Phosphorylation of S473 is inhibited by tribble homologue 3 (TRB3) and C-terminal modulator protein (CTMP). Also PDK1 contains a PH domain, which mediates its membrane localization upon elevated PtdIns(3,4,5)P_3 levels. Phosphorylation of PKB leads to its activation and translocation from the membrane (Figure 6). Many downstream targets of PKB (most of them sharing a R-X-R-X-X-S/T-φ[1] consensus motif) have been characterized. Among them we find proteins like BAD, CREB, GSK3, mTOR, p21 or Raf. For reviews see also (Datta *et al.*, 1999; Brazil and Hemmings, 2001; Brazil *et al.*, 2004). As already mentioned, evidence for a role of PKB in stabilization of ARE-mRNA via inactivation of BRF1 has been published recently by our group (Schmidlin *et al.*, 2004) and will be presented here.

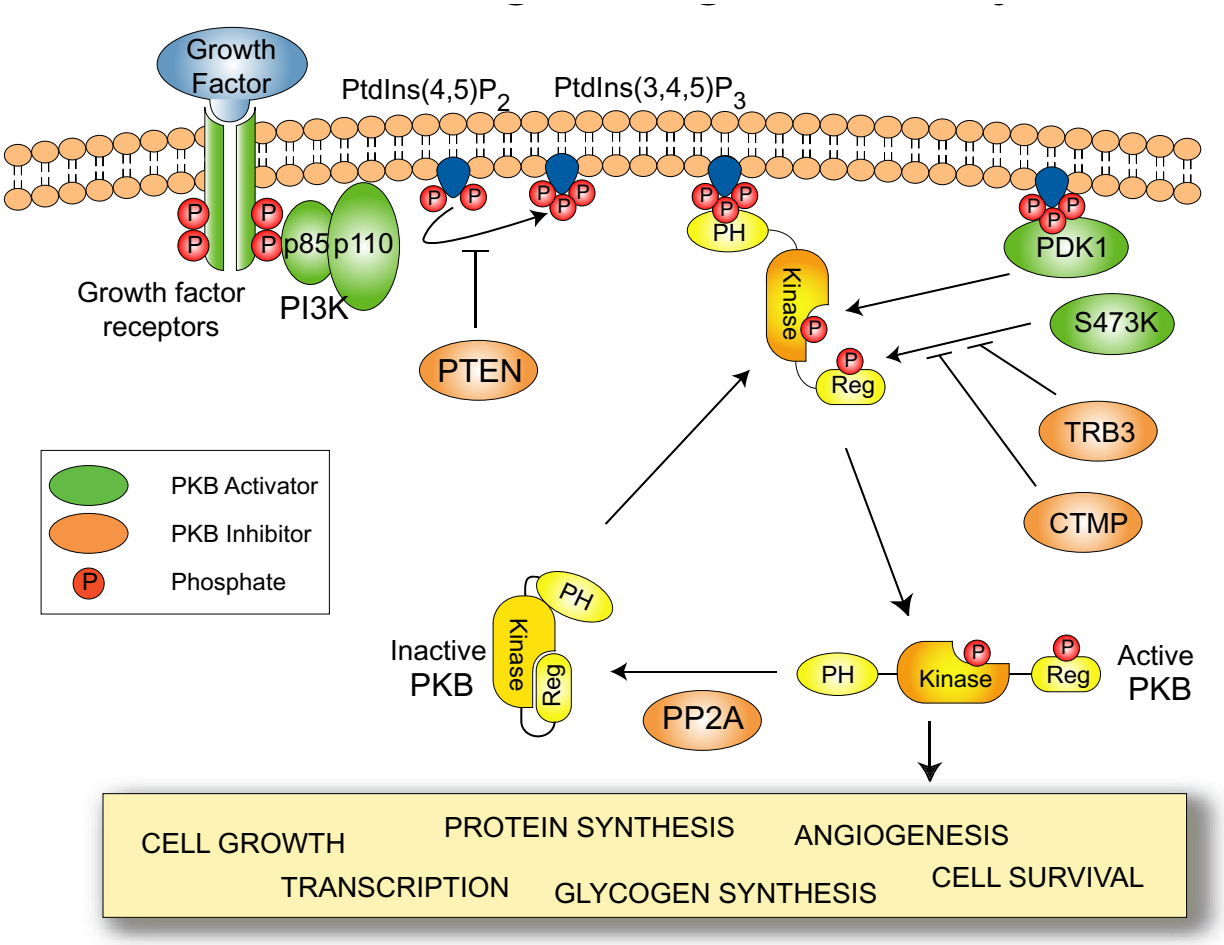


Fig. 6: Overview on PKB signaling, adapted from (Brazil *et al.*, 2004). Some of the most important biological responses of this pathway are mentioned.

[1] X: any amino acid/ φ:hydrophobic amino acid

Protein Kinase C (PKC)

PKC also belongs to the AGC family of serine-threonine kinases. For activation, PKC needs to be phosphorylated by PDK1 resulting in an autophosphorylation step. PDK1 activity is regulated by PI3-K via the levels of PtdIns(3,4,5)P_3 as described in the previous chapter. In the enzymatically competent (phosphorylated) state PKC is ready for activation by second messengers: The classical PKC isoforms are activated by calcium, diacylglycerol (DAG) and phospholipids, the novel isoforms by DAG and phospholipids, whereas the atypical isoforms can only be activated by phospholipids. A signaling cascade initiated by G-protein coupled receptors (GPCR) activates phospholipase C (PLC), which leads to the production of DAG, inositol-1,4,5-trisphosphate (IP_3) and release of calcium. Phorbol-esters (e.g. TPA) are strong activators of some PKC isoforms, as they mimic the actions of DAG. Upon full activation PKC can then activate ERK1/2 and Raf. (reviewed in (Liebmann, 2001; Spitaler and Cantrell, 2004)).
TPA is a potent stabilizer of ARE-mRNA, indicating that PKC is also involved in the control of mRNA turnover (Ming *et al.*, 2001).

Small G-Protein ras

The ras genes were originally found in the 1960s as the transforming elements of the Harvey and Kirsten strains of rat sarcoma viruses. Later mutated alleles of cellular ras genes were identified as dominant oncogenes in various human tumors. The mammalian ras family consists of three members. The H- and K-ras genes are the human homologs of viral genes and N-ras is derived from a human neuroblastoma cell line (reviewed in (Bar-Sagi, 2001)). Recently a ras like gene, expressed in embryonic stem cells, was cloned. This E-ras protein contains amino acids identical to the active mutants of ras and is oncogenic when transfected into 3T3 cells (Takahashi *et al.*, 2003). The ras proteins are anchored to the plasma membrane via a farnesyl and/or palmitoyl modification. ras exists in two forms: a GTP bound, active and a GDP bound, inactive form. Most stimuli activate ras by recruiting proteins that exchange GDP with GTP (so called GDP/GTP exchange factors (GEFs)) to the membrane associated ras protein. Activation of RTKs leads to recruitment of growth-factor-receptor bound protein 2 (Grb2), serving as an adaptor protein for SOS (son of sevenless), the ras GEF. Three main classes of effector proteins are primarily stimulated by ras: Raf kinases (see ERK-pathway), PI3-K (discussed above) and RalGEFs (reviewed in (Hilger *et al.*, 2002)).
ras is a central player in many pathways and it has been shown that transfection of activated ras efficiently stabilizes ARE-mRNA. This effect is dominant over any destabilizing function of BRF1 or TTP (Sabrina Leuenberger, PhD thesis 2004).

Mitogen Activated Protein Kinase (MAPK) Pathways

MAPKs are evolutionary conserved enzymes, connecting extracellular signals, recognized by cell surface receptors, to the appropriate intracellular target molecules. MAPK cascades consist of three core components: A MAPK, activated by a MAPK kinase (MAPKK), which again is activated by a MAPKK kinase (MAPKKK). Four MAPK cascades are known: The ERK1/2, JNK, p38 (described below) and ERK5 pathways. MAPKs are involved in regulation of a vast variety of cellular processes, ranging from gene expression, mitosis, cell-movement and metabolism to apoptosis.

Although individual functions have been assigned to different MAPK cascades, there is substantial crosstalk between the different kinases (see Figure 7). For review see (Chang and Karin, 2001; Johnson and Lapadat, 2002).

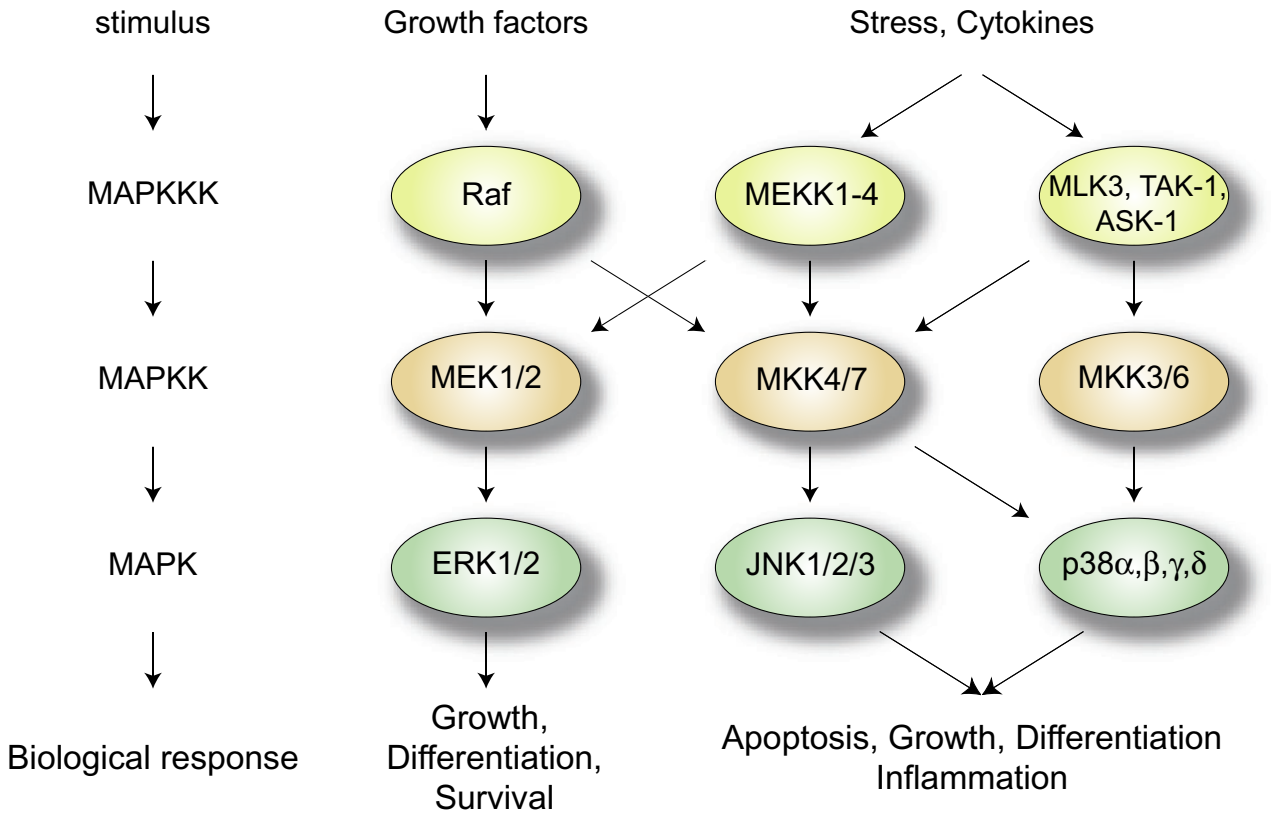


Figure 7: MAPK-pathways. Some of the most important biological responses of these pathways are mentioned. MAPK: Mitogen activated protein kinase, MAPKK: MAPK kinase. MAPKKK: MAPKK kinase.

ERK Pathway

ERK1 and ERK2, the two MAPKs in the ERK pathway are widely expressed. They are involved in differentiation, mitosis, meiosis and development. The Raf-MEK-ERK pathway is the best characterized MAPK signaling pathway. This signaling cascade is activated either through stimulation of RTKs or GPCRs by cytokines, growth factors, by virus infection or transforming agents. In the case of GPCR, the signal is then transduced via PKC or PKA. Raf (the MAPKKK in this cascade), which can be activated by the small G-protein ras, signals to MEK1/2 (MAPKKs), which leads to activation of ERK1/2 (MAPK). Activated ERK then translocates to the nucleus, where transcription factors are the substrates, leading to expression of genes in response to the original stimuli. Among them are Elk1, myc and STAT1/3 (Hilger *et al.*, 2002; Chong *et al.*, 2003). Recently it was shown by Yang *et al.* that prostaglandin A2 mediates stabilization of the cyclin-dependent kinase inhibitor p21 mRNA. Inhibition of the ERK pathway did inhibit this stabilization and the formation of a HuR-p21 mRNA complex, implicating a role of the ERK pathway in regulation of HuR activity (Yang *et al.*, 2004). *In vitro* phosphorylation assays have shown that ERK2 phosphorylates TTP at S220 (Taylor *et al.*, 1995). This site seems to be a major site of mitogen-

stimulated TTP phosphorylation. However, no follow-up publications attributed a functional role to this phosphorylation.

JNK (c-jun N-Terminal Kinase) Pathway

The JNK signaling pathway is activated by several different MAPKKKs including members of the MEKK group, the ASK1 group, TAKI and MLK3. The signal is transduced via MKK4 and MKK7. Activated JNK binds to and phosphorylates the N-terminal activation domain of *c-jun*. Trans activation of *c-jun* increases expression of genes with AP-1 sites in their promoters e.g. *c-jun* itself, ATF-2, Elk1, p53 and others. JNKs activation is involved in proliferation, differentiation, apoptosis and survival signaling.

Activation of stress-induced JNK was shown to stabilize ARE-mRNA (Chen *et al.*, 1998; Ming *et al.*, 1998). Interestingly, in mast cells JNK mediates ionomycin-induced stabilization of ARE-mRNA, but activation of JNK in NIH3T3 cells has no effect on mRNA stability (Ming *et al.*, 2001). This indicates that, dependent on the cell type, ARE-mRNA stability is controlled by different pathways.

p38 MAPK Pathway

There are four p38 kinase isoforms α, β, γ and δ, with p38α being the best characterized. p38 MAPKs are key regulators of inflammatory cytokines and play an important role in immune response. Various stress signals such as UV-irradiation, LPS, inhibition of protein synthesis, osmotic- or heat-shock activate the p38 MAPK signaling cascade. The signals are transduced via MEK3/6 (MAPKK) to p38. Interestingly, p38 has also been found to be activated by TAB1, which is not an MAPKK but rather a scaffold protein without known catalytic activity. This indicates that in addition to the MAPK signaling modules other regulatory mechanisms exist (reviewed in (Johnson and Lapadat, 2002)).

p38 has been reported by several groups to be involved in stability control of ARE-mRNA (Dean *et al.*, 1999; Winzen *et al.*, 1999; Brook *et al.*, 2000; Clark *et al.*, 2003; Frevel *et al.*, 2003; Tchen *et al.*, 2004; Winzen *et al.*, 2004). HuR has been shown to act in concert with p38 in stabilizing IL-3 mRNA (Ming *et al.*, 2001), although direct phosphorylation of HuR was not detected. TTP, on the other hand, can be phosphorylated by p38. This phosphorylation inhibits its ARE binding activity (Carballo *et al.*, 2001; Zhu *et al.*, 2001). Also MAPK activate protein kinase 2 (MAPKAPK2 or MK2), a downstream kinase of p38, has been implicated in RNA stabilization (Neininger *et al.*, 2002). MK2 is able to phosphorylate TTP *in vitro* (Mahtani *et al.*, 2001). MK2 mediated phosphorylation of TTP could be mapped to S54 and S178 and was found to facilitate 14-3-3 binding. 14-3-3-interaction inhibits TTP activity and leads to exclusion of TTP from stress granules (Johnson *et al.*, 2002; Chrestensen *et al.*, 2003; Stoecklin *et al.*, 2004). In contrast to these data, phosphorylation of TTP by MK2 has also been shown to be required for efficient TNFα-ARE binding (Mahtani *et al.*, 2001).

Different MAPK pathways seem to converge on TTP. Cao and coworkers could show that recombinant TTP is phosphorylated by p38, ERK2 and JNK, however, none of these phosphorylations did affect RNA binding (Cao *et al.*, 2003). Further, Ming *et al.* (Ming *et al.*, 2001) in our lab showed that TTP

antagonizes ARE-mRNA stabilization by p38 and PI3-K, indicating that TTP is not inactivated by phosphorylation through these pathways. The data summarized here does still not satisfyingly explain the functional role of TTP phosphorylation. Further experiments are needed to dissect the effects of all these pathways on AUBPs.

14-3-3 Proteins

The 14-3-3 proteins are involved in a multitude of cellular processes such as cell cycle control, stress response and apoptosis. They have been found in all eukaryotic organisms ranging from yeast (2 isoforms) to mammals, where seven isoforms have been described (β, γ, ε, σ, ζ, τ and η). 14-3-3 proteins were the first proteins described to bind specifically to phospho-serines in one of the following motifs: R-S-X-pS-X-P (mode 1) or R-X-X-X-pS-X-P (mode 2) (Yaffe *et al.*, 1997). In the last few years over 200 interacting proteins have been identified (Rubio *et al.*, 2004), reflecting the complexity of 14-3-3 function. Well-characterized examples are Raf-1, BAD, or Cdc25. 14-3-3-interaction can affect the localization of proteins, their enzymatic activity, DNA-protein or protein-protein interactions (reviewed in (van Hemert *et al.*, 2001; Tzivion and Avruch, 2002; Mackintosh, 2004). Many PKB targets bind to 14-3-3 upon phosphorylation, as the PKB consensus sequence (R-X-R-X-X-S/T-φ) may overlap the optimal 14-3-3 binding sequence (Tzivion and Avruch, 2002).
MK2 induced interaction of TTP with 14-3-3 has already been discussed in the previous chapter. 14-3-3 also interacts with BRF1, as suggested by results from a yeast two-hybrid screen (Bustin and McKay, 1999) and from GST-pulldown experiments (Johnson *et al.*, 2002). Recently, we were able to show that phosphorylation of BRF1 by PKB inactivates its decay promoting activity probably by binding 14-3-3 (Schmidlin *et al.*, 2004).

Wnt Pathway

The Wnt signaling cascade induces a wide range of responses from cell proliferation to differentiation. Extracellular Wnt signals interact with members of the Frizzled family of serpentine receptors. These activate the cytoplasmic Dishevelled (Dvl) protein, which inhibits the constitutive proteasomal destruction of β-catenin. β-catenin accumulates in the nucleus, where it interacts with DNA-bound TCF and LEF family of transcription factors to activate transcription of target genes (for review see (Moon *et al.*, 2004)).
Briata *et al.* showed that activation of the Wnt-β-catenin pathway not only induces transcription of Pitx2, a transcription factor regulating genes, such as *c-jun*, cyclin D1 and D2, but also stabilizes the Pitx2, *c-jun*, cyclin D1 and D2 mRNA. The stabilization is probably achieved by reducing the interaction of the ARE of these mRNAs with the destabilizing proteins KSRP and TTP and by facilitating the interaction of the RNA with the stabilizing HuR (Briata *et al.*, 2003).

Regulation of mRNA Stability

As summarized above, activation of many signaling pathways leads to stabilization of ARE-containing mRNA. The different signals seem to converge on the AUBPs. The sum of the incoming signals decides over the fate of the mRNA. Due to the multitude of signaling pathways and AUBPs, tight regulation of the mRNA turnover can be achieved.

In addition to the control via AUBPs, mRNA turnover seems to be coupled to translation. Mutations in translation initiation factors lead to increased rates of deadenylation and decapping of yeast mRNA (Schwartz and Parker, 1999).
Recent unpublished data presented at the annual RNA conference of the RNA society 2004 show that stabilization of ARE-mRNA involves reduction of PARN activity via proteasomal degradation. This effect seems to be mediated by AUBPs (Biswas *et al.* abstract 454). Further, BRF1 and TTP have been shown to interact with the Dcp1/2p decapping complex and with the hCcr4d-hCaf1z deadenylation complex (Lykke-Andersen and Wagner, 2005). In the case of TTP the binding activity was mapped to the N-terminal domain. Further, it has been shown that the cap binding protein eIF4G interacts with the poly(A) binding protein PABP (Imataka *et al.*, 1998; Tarun and Sachs, 1996). This leads to model, where translation and mRNA degradation are tightly coupled. The mRNA forms a circular structure with multiple protein complexes interacting with different regions of the mRNA (e.g. cap, ARE or poly(A)-tail) and with each other. The composition and the exact structure of these complexes, which seems to be regulated by external stimuli, determine the fate of the bound RNA.

The Project(s)

The project(s) aimed at understanding, how signal transduction pathways regulate the activity of AUBPs, and subsequently affect mRNA turnover. The main focus of this work was laid on BRF1, as very little was known about its regulation. Four different points were addressed during these studies:

Regulation of BRF1 by Phosphorylation at Serine 92. First, the regulation of BRF1 by phosphorylation was investigated. *In silico* analysis of the protein sequence of BRF1 revealed many putative phosphorylation sites. Serine 92 (S92), which is placed in the context of a PKB consensus site, was already shown by mass spectrometry to be phosphorylated by PKB *in vitro* (Schmidlin *et al.*, 2004). This modification was now investigated *in vivo*. To achieve this, a phospho-S92-specific antibody was purified, characterized and used for detection of *in vivo* phosphorylation. The stability of ARE-containing message was investigated under conditions of BRF1 phosphorylation.

*Regulation of BRF1 by Phosphorylation at sites different from S92***.** Second, the question was addressed, whether other kinases than PKB are involved in regulating BRF1 activity, as it is suggested by the presence of the additional putative phosphorylation sites. Arsenite- (in NIH3T3 cells) and insulin-treatment (in HIRc-B rat fibroblasts, overexpressing the human insulin receptor) was used to induce hyperphosphorylation of BRF1. This could be visualized by a shift in electrophoretic mobility of the protein. In an attempt to identify the pathways involved, different inhibitors were tested for their ability to inhibit hyperphosphorylation of BRF1. Comparison of the sequences of TTP and BRF1 revealed S203 as the homologous site of S178 in TTP. S178 in TTP is known to be phosphorylated by MK2 and facilitate 14-3-3 binding. Therefore, S203 was chosen for more detailed analysis. The serine to alanine mutant (S203A) was compared to the wild-type protein in respect to electrophoretic mobility and decay promoting activity.

Localization of AUBPs. In a third part, the subcellular localization of the four AUBPs HuR, AUF1, BRF1 and TTP was investigated, as nucleo-cytoplasmic shuttling of these proteins might be part of their regulation. The questions were asked, whether changes in stability of ARE-containing mRNA involves changes in localization of AUBPs and whether stabilizing and destabilizing proteins are differently regulated. By transfection of activated kinases or by drug treatment, specific pathways could be turned on and their effect on localization of the AUBPs was analyzed by immunofluorescence.

Involvement of BRF1 in Cell Cycle Control. The last part of the work was aimed at investigating the physiological role of BRF1. ARE-mRNA turnover has been shown to play a role in cell cycle control. Therefore, the question was addressed, whether BRF1 plays a role in this process. For this purpose, a cell line with doxycycline-repressible BRF1 expression was established. Cell cycle progression of synchronized cells was analyzed by propidium iodide staining followed by flow cytometry and by [^{3}H]-thymidine incorporation. Cells with physiological BRF1 levels were compared to cells overexpressing BRF1 and to cells with reduced BRF1 expression (RNAi).

RESULTS

The results section of this thesis is divided into four parts. First, after a summary of previous data from the lab, leading to this work, the *in vivo* phosphorylation of BRF1 at S92 will be discussed. In a second part, additional putative phosphorylation sites in BRF1 will be analyzed. Third, the localization of BRF1 and three other AUBPs (TTP, HuR and AUF1) will be investigated and in a fourth part the question will be addressed, whether BRF1 plays a role in cell cycle regulation.

BRF1 is Phosphorylated at S92

Previous Work from the Laboratory

Several signal transduction pathways have been found to participate in the regulation of ARE-mRNA stability. Previous work in our lab started by Ming *et al.* showed that both the PI3-K and the p38 kinase pathway are independently involved in stabilization of ARE-mRNA (Ming *et al*., 2001). Subsequent work by Sabrina Leuenberger showed that PKB, a downstream element of PI3-K, stabilizes ARE-mRNA, indicating that the stabilizing effect of PI3-K is exerted via this axis. As mentioned in the introduction, some AUBPs have been shown to be direct targets of signaling pathways. TTP, for instance, is phosphorylated by p38 (Carballo *et al*., 2001; Zhu *et al*., 2001), by the p38 downstream kinase MK2 (Stoecklin *et al.*, 2004; Chrestensen *et al*., 2003), by JNK (Cao *et al.*, 2003) and by ERK2 (Taylor *et al.*, 1995). Our lab is mainly interested in BRF1, which was functionally cloned by Georg Stoecklin and Marco Colombi (Stoecklin *et al*., 2002). In the case of BRF1 no regulatory events have been described, when this study was started. Therefore, the question was asked, whether BRF1 activity is regulated via direct phosphorylation, similar to TTP, and if so, which signaling pathways are involved in this regulation.

The protein sequences of murine and human BRF1 (SwissProt database; accession number P23950 and Q07352, respectively) contain consensus motifs for many putative phosphorylation sites (Figure 8/ http://scansite.mit.edu/ Obenauer *et al*., 2003). Serines 90 and 92 (S90, S92) are placed in the context of two overlapping PKB consensus sites (R-X-R-X-X-S-ϕ (Obata *et al*., 2000)). In the lab of Brian Hemmings at the Friedrich Miescher Institute in Basel it could be shown by mass-spectrometry of *in vitro* phosphorylated BRF1 that S92 is efficiently phosphorylated by PKB (Schmidlin *et al*., 2004). To further study the function and the regulation of BRF1, recombinant BRF1 (rBRF1) was tested in an *in vitro* decay system. This approach has successfully been used by others (Ford *et al.*, 1999; Chen *et al.*, 2000; Gao *et al.*, 2001) to decipher different aspects of post-transcriptional

Figure 8: Putative phosphorylation sites in human (huBRF1) and murine BRF1 (muBRF1) are shown in red (sequences were retrieved from the SwissProt database; accession number P23950 and Q07352, respectively, and analyzed on http://scansite.mit.edu)).

regulation of mRNA. Indeed, addition of rBRF1 to slowC cell extracts, lacking BRF1 expression, due to frameshift mutagenesis (Stoecklin *et al*., 2002), did promote degradation of ARE-containing RNA. Interestingly, phosphorylation of BRF1 at S92 inhibited its decay promoting activity in the same assay. A mutant version of BRF1, with S90 and S92 replaced by alanine (BRF1S90/92A) was refractory to this inhibition, indicating that S92 is important for PKB mediated inactivation of BRF1. (As residual phosphorylation of the S92A mutant was observed in *in vitro* kinase assays, the double mutant S90/92A was routinely used, to circumvent unspecific phosphorylation of S90). Looking for a possible mechanism to explain the inhibition of BRF1 by phosphorylation, the attention was turned to the 14-3-3 protein, which is known to be involved in many signaling events.
14-3-3 interacts with more than 250 different proteins, mostly in a phosphorylation dependent manner (Meek *et al*., 2004; Rubio *et al*., 2004). Among them we find many PKB targets. One hypothesis, therefore, was that 14-3-3 binds to phosphorylated BRF1 and, thereby, inhibits its activity. Indeed, it

could be shown that rBRF1 can pull down 14-3-3 from cellular extracts only when pre-treated with activated PKB. Further, 14-3-3 co-purifies with His-tagged BRF1, but not with BRF1S90/92A from Cos7 cells upon activation of the PKB signaling pathway (Schmidlin *et al*., 2004).

Purification and Characterization of a phospho-S92-BRF1 Antibody

So far, the findings were based on *in vitro* experiments. However, it is well known that phosphorylation observed *in vitro* may not reflect the physiological situation. To investigate, whether S92 is a regulatory site *in vivo*, an antibody directed to a trisdekapeptide containing phosphorylated S92 of BRF1 (F-R-D-R-S-F-pS-E-G-G-E-R-L) was raised in rabbits (immunization of the rabbit was performed by Brigitte Gross). I then tested the sera of three rabbits for their ability to detect rBRF1, pre-incubated with active PKB, on Western blot. One serum (#6) tested positive and was specific for phosphorylated BRF1, as untreated rBRF1 did not give rise to a Western signal (Figure 9A, compare lanes 10 and 11). For purification, serum #6 was run over an affinity Sepharose® 4B column (Pharmacia Biotech) with the phospho-peptide, used for immunization, coupled to the beads. After washing, the antibody was eluted from the column at pH 2,6. The eluate was fractionated in 1ml aliquots and neutralized to pH 7. The fractions were tested again on Western blot for their ability to distinguish between phosphorylated and unphosphorylated

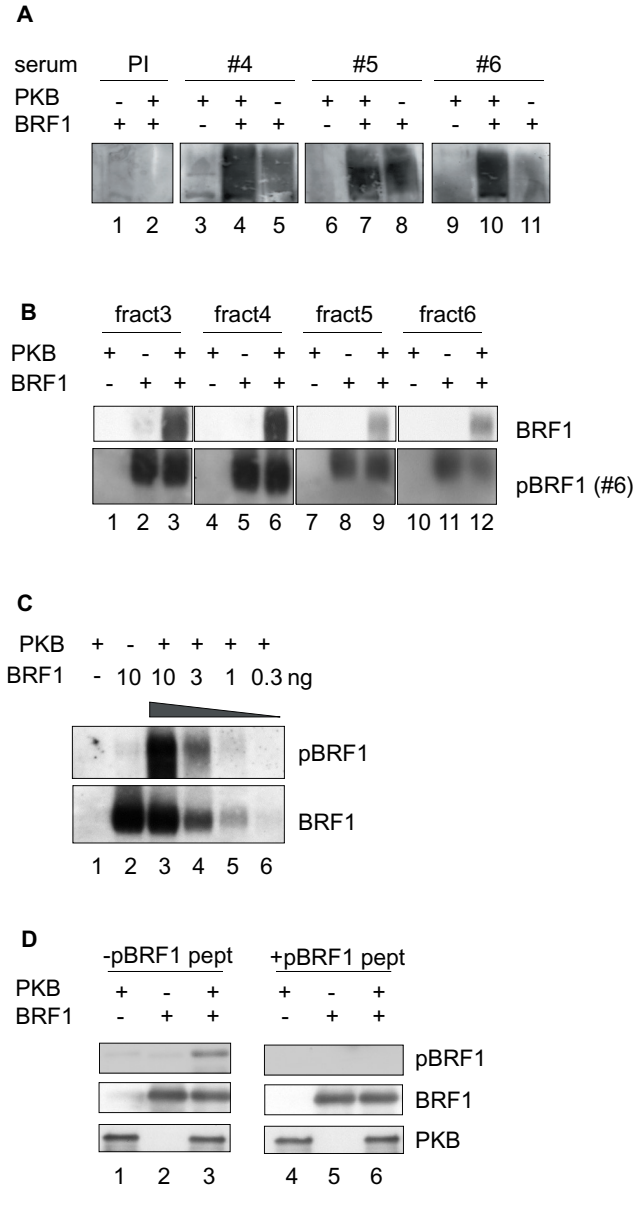


Figure 9: **A:** rBRF1 was incubated alone or with activated PKB. Pre-immun serum (PI) and the 3 different sera (1/10 diluted) from the immunized rabbits (#4, #5, #6) were tested on Western blot for their specific recognition of phosphorylated BRF1. 10ng of BRF1 protein were loaded. **B:** Affinity purified fractions 3 to 6 from serum #6 (1/200 diluted), were tested as in A. As a loading control the same blot was probed with a rabbit anti-BRF1 antibody (Raineri *et al*., 2004). **C:** rBRF1 was pre-incubated with activated PKB and indicated amounts were processed for Western blot. Phosphorylated rBRF1 was detected with serum #6, fraction 4 (1/200). **D:** Where indicated (+pBRF1 pept.), the antibodies recognizing BRF1 and phospho-S92-BRF1 were pre-incubated with 50µg of the phospho-peptide used for immunization (F-R-D-R-S-F-pS-E-G-G-E-R-L) before immunodetection. As an additional control PKB levels were detected.

rBRF1 (Figure 9B). Fraction 4 gave a strong signal in the case of phosphorylated rBRF1 and did not detect unphosphorylated rBRF1 (compare lanes 5 and 6). This fraction was further characterized. Titration of rBRF1 revealed that approximately 1ng of phosphorylated rBRF1 could still be detected (Figure 9C, lane 5) and that the specificity for phosphorylated rBRF1 was about 10-fold higher than for unphosphorylated rBRF1, because 10ng unphosphorylated rBRF1 gave raise to a comparable signal to 1ng of phosphorylated rBRF1 (Figure 9C, compare lanes 2 and 5). As an additional control for its specificity, the antibody was pre-incubated with the phospho-peptide used for immunization before immunodetection. The phospho-signal could be competed away (Figure 9D, compare lanes 3 and 6), whereas pre-incubation of the antibody, recognizing the C-terminus of BRF1 (Raineri *et al*., 2004), with the same peptide had no effect (middle panel, compare lanes 2, 3 with 5, 6).

Phosphorylation of S92 by Transfected PKB

Having characterized the phospho-S92-BRF1 antibody, the question was addressed, whether BRF1 is phosphorylated at S92 *in vivo* and whether PKB is, indeed, the kinase responsible for this modification. For this purpose wild-type BRF1 (BRF1wt) and BRF1S90/92A (again the double mutant was used to avoid residual phosphorylation of S90) were transfected together with activated m/pPKB (Andjelkovic *et al*., 1997) or kinase dead PKB (kdPKB) into NIH3T3 cells. m/pPKB is a myristoylated/palmitylated form of PKB that is permanently localized to the plasma membrane and therefore constitutively activated (see Introduction, Figure 6). 24h after transfection, the samples were processed for Western blotting and phospho-S92-BRF1 (pBRF1), phospho-S473-PKB (pPKB, activated PKB) and α-tubulin (α-tub), as a loading control, were visualized by the appropriate antibodies. As seen in Figure 10A, transfection of BRF1wt together with kdPKB gave rise to a weak signal, detected with the phospho-S92-BRF1 antibody. This band was absent, if BRF1S90/92A was transfected (Compare lanes 1 and 2), indicating that the signal is indeed specific for phosphorylated S92. The phospho-S92-BRF1 antibody detects also an unspecific band, migrating slightly above BRF1. The specific size for BRF1 is indicated by the arrow. Cotransfection of highly active m/pPKB (see middle panel, lanes 3 and 4) did strongly increase the phosphorylation of S92, with no effect on BRF1S90/92A (lanes 3, 4). This experiment shows that PKB does indeed phosphorylate BRF1 at S92 *in vivo*. However, these data do not clarify, whether the weak phosphorylation detected in lane 1 is due to background activity of endogenous PKB (which is very weak, as seen in the middle panel) or another kinase.

The same experiment was also performed in HIRc-B cells (kindly provided by P. Blackshear (McClain *et al*., 1987)). These rat fibroblast cells overexpress the human insulin receptor, rendering them hypersensitive to insulin stimulation. HIRc-B cells were chosen in prospect of future experiments, using insulin for PKB activation. In addition to the experiment described in Figure 10A, BRF1wt and BRF1S90/92A were also transfected alone and PKB activity was inhibited with wortmannin (WM, a PI3-K inhibitor) to test, whether background PKB activity can account for the phosphorylation of exogenous BRF1. The levels of phospho-S92-BRF1 (pBRF1), BRF1, active PKB (pPKB), total PKB and α-tubulin (α-tub), as a loading control, were visualized. The BRF1 antibody recognizes in addition to BRF1 also BRF2, which shares the C-terminal peptide used for immunization. The two proteins can be separated by size. RNAi experiments had previously revealed that the upper band, indeed, corresponds to BRF2 (Brigitte Gross, unpublished data).

Interestingly, the results clearly showed that the phosphorylation of BRF1 at S92 under these conditions is WM-insensitive, as treatment with WM, which completely blocked PKB activation (middle panel, compare lane 1 and 2) did not reduce the phosphorylation of BRF1 at S92 (Figure 10B, compare lane 1 and 2, upper panel). To assure that the signal detected by the phospho-S92-BRF1 antibody is indeed phospho-specific, BRF1S90/92A was analyzed. As expected, the phospho-BRF1 signal was strongly reduced in this case (lanes 3 and 4, upper panel). Again the topmost band in the upper panel is unspecific. Transfection of m/pPKB alone did lead to high levels of activated PKB (lane 5, middle panel) and phosphorylated endogenous BRF1 could be detected (upper panel), despite the low levels of BRF1 (second panel from top). Transfection of kinase dead PKB (kdPKB) had no effect on BRF1 phosphorylation (lane 6, upper panel).
Cotransfection of BRF1wt or BRF1S90/92A together with m/pPKB or kdPKB resulted in a similar pattern to the one seen in NIH3T3 cells. Again a background phosphorylation of BRF1 could be detected, if kdPKB was cotransfected with BRF1wt (Figure 10B, lane 7), which increased substantially, if the activated form of PKB was transfected (compare lanes 7 and 9). Phosphorylation was specific to S92, as no phosphorylation of the BRF1S90/92A mutant could be detected under either condition. These data confirm the finding that BRF1 is phosphorylated *in vivo* by PKB at S92 and show that at least one WM-independent kinase can also phosphorylate BRF1 at this same site.

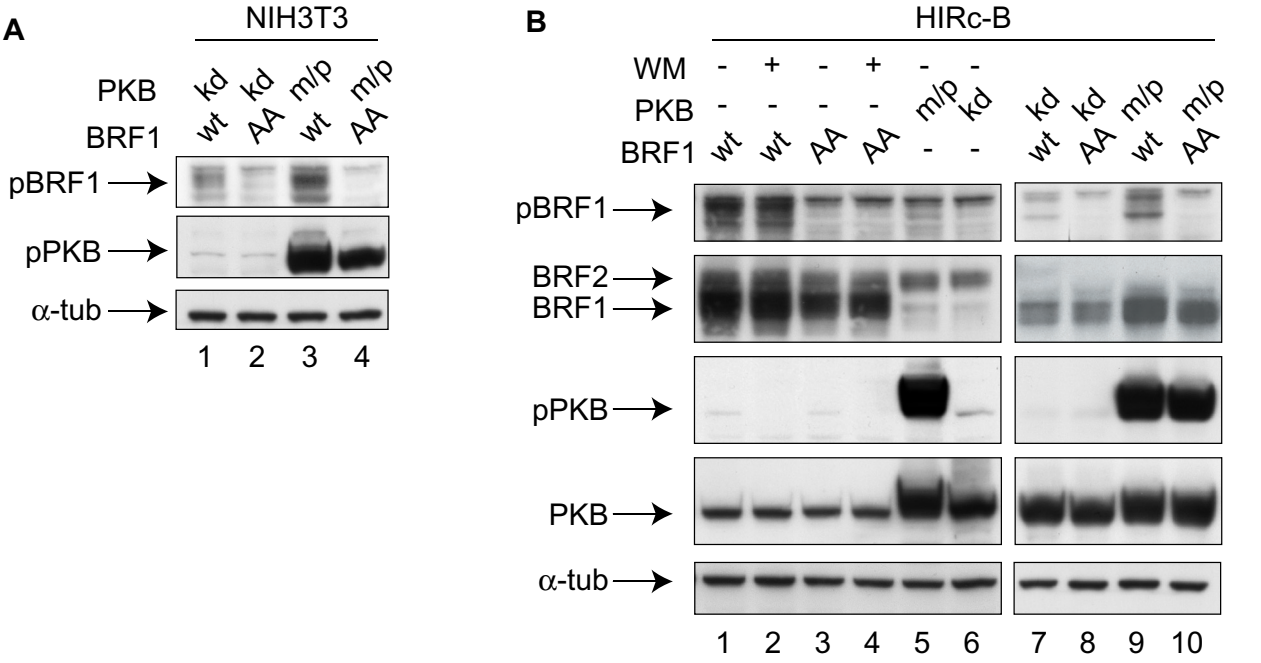
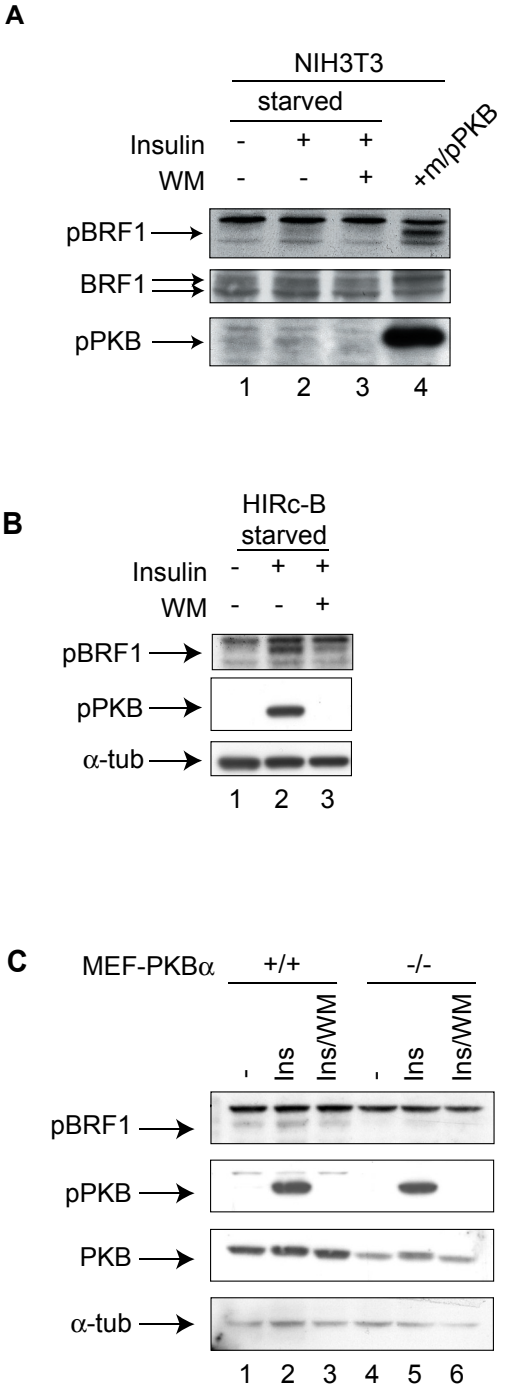


Figure 10: **A:** NIH3T3 (B2A2 clone23 (Xu *et al*., 1998)) were transfected with bsd-HisBRF1wt (wt), bsd-HisBRF1S90/92A (AA), activated PKB (m/p) or PKB kinase dead (kd), as indicated. 24h after transfection, cellular extracts were processed for Western blotting and phosphorylated BRF1 at S92 (pBRF1), activated PKB (pPKB) and α-tubulin were detected. **B:** HIRc-B cells were transfected with bsd-HisBRF1wt (wt), bsd-HisBRF1S90/92A (AA), activated PKB (m/p) or PKB kinase dead (kd) alone or in combination. After WM addition (200nM, 45min), where indicated, extracts were processed for Western blotting and phospho-S92-BRF1 at S92 (pBRF1), total BRF1, BRF2, activated PKB (pPKB), total PKB, and α-tubulin were detected.

Phosphorylation of S92 in BRF1 by Insulin

While the data above established that PKB is able to phosphorylate S92, it has to be considered that the levels of activated PKB following transfection are unphysiologically high. To further investigate the significance of S92 phosphorylation under more physiological conditions, NIH3T3 cells were treated with insulin, a known activator of PKB (Burgering and Coffer, 1995). PKB background activity was reduced, by starving the cells overnight in serum-free medium. The phosphorylation status of BRF1 was analyzed by Western blot together with total BRF1 levels and levels of activated PKB (pPKB). As a control, m/pPKB transfected extract was run on the same gel. As expected, m/pPKB strongly activated PKB (Figure 11A, lane 4, lower panel). Under the same conditions BRF1 phosphorylation was induced (lane 4, upper panel) with no change in BRF1 protein levels (lane 4, middle panel). Also after insulin-treatment for 15 minutes a weak phospho-S92-BRF1 band was seen (upper panel, middle band), however, PKB activation was only minimal as revealed by detection of phospho-S473-PKB (Figure 11A, lane 2, lower panel). Nevertheless, PKB seemed to be the kinase responsible for this phosphorylation, as the phospho-S92-BRF1 signal disappeared, if the cells were pre-treated with the PI3-K inhibitor wortmannin (WM) for another 30' prior to insulin addition (compare lanes 2 and 3, upper panel).



To verify these findings and to increase the insulin induced PKB activation, further insulin stimulation experiments were performed in HIRc-B rat fibroblasts, which highly express the human insulin receptor and are widely used to investigate insulin signaling (McClain *et al*., 1987). In these cells, insulin strongly activated PKB (Figure 11B, lane 2, middle panel). Phosphorylation of BRF1 at S92 could be detected under conditions of PKB activation. In contrast to NIH3T3 cells, in HIRc-B cells phosphorylation

Figure 11: **A:** NIH3T3 cells were starved overnight and stimulated with insulin (20µg/ml, 15min) after pretreatment of WM (200nM, 30min) as indicated. As a control NIH3T3 cells transfected with m/pPKB were analyzed on the same blot. BRF1 phosphorylated at S92 (pBRF1), total BRF1 and activated PKB (pPKB) levels were analyzed. **B:** HIRc-B cells were treated as described in A. In addition α-tubulin was detected as a loading control. **C:** MEF-PKBα +/+ and -/- cells were treated as described in A. pBRF1, pPKB, total PKB and α-tubulin levels were analyzed

was not completely abolished by pre-treating the cells with WM, which completely blocked PKB activation (compare lanes 2 and 3). Again, this result indicates that, in addition to PKB, another kinase is able to phosphorylate S92. To further strengthen the point that PKB phosphorylates BRF1 at S92 *in vivo*, the effect of insulin stimulation was tested in mouse embryonic fibroblasts lacking PKBα (MEF-PKBα -/- (Yang *et al.*, 2003) kindly provided by Brian Hemmings). Again, these cells and the corresponding wild-type control cells were starved overnight and then treated for 30 minutes with WM, before insulin was added for 15 minutes. As expected, the MEF-PKBα -/- cells showed reduced levels of PKB (Figure 11C, third panel, lanes 4-6). The antibody used for PKB detection cross reacts with all three PKB isoforms. The residual signal, therefore, arises form the β and γ isoform still present in the knockout cells. Similar to the wild-type cell also the knockout showed activated PKB after insulin stimulation, which was inhibited by WM, again arising from the other two isoforms. In the wild-type cells, a basal BRF1 phosphorylation could be detected (lane 1, upper panel), which was increased upon insulin stimulation (lane 2) and again reduced by WM (lane 3). Interestingly, BRF1 phosphorylation was absent in the MEF-PKBα -/- cells and only marginally induced upon insulin stimulation (compare lanes 4 and 5). These data indicate that PKBα is responsible for insulin induced BRF1 phosphorylation in MEFs.

ARE-mRNA is Stabilized under Conditions of BRF1 Phosphorylation

To test the effect of BRF1 phosphorylation and to check, whether *in vivo* PKB mediated BRF1 phosphorylation inhibits the decay promoting activity of BRF1 similar to the *in vitro* situation, the stability of ARE-mRNA was investigated upon stimulation of HIRc-B cells by a physiological stimulus, such as insulin. For this purpose, a doxycycline controllable reporter system was chosen: The tetracycline-controlled transactivator (tTA), a fusion protein of the tetracycline (tet) repressor (tetR) and the c-terminal activation domain of the VP16 protein of herpes simplex virus, can bind to the tet operator sequence (tetO) present in the tet responsive element (TRE). If the TRE is placed next to a transcriptionally silent minimal CMV promoter lacking the enhancer (P_{minCMV}), binding of tTA leads to transactivation. In the presence of doxycycline (dox) or tet tTA-TRE interaction is inhibited and transcription is silenced (Figure 12).

HIRc-B cell were cotransfected with the tTA transactivator and a dox-controllable β-globin reporter construct with the 3'UTR of IL-3, containing a strong class II ARE. The cells were transfected in a 15cm dish and splitted into three 10cm dishes 24h after transfection. After another 24hrs, the cells were treated for 30min with WM, followed by 15min insulin, where indicated, and then transcription was stopped by addition of dox. RNA levels of the β-globin-IL3-UTR construct were analyzed by northern blot. In unstimulated cells the reporter message was degraded with a half-life of approximately 2h (Figure 13A, lanes 1-3 and quantification). Insulin stimulation did stabilize the message significantly (lanes 4-6) and WM pre-treatment reversed this stabilizing effect (lanes 7-9).

To underline the importance of the regulation of BRF1 by PKB, I sought to find an endogenous target mRNA, which is regulated similar to the reporter construct. The attention was turned to vascular endothelial growth factor (VEGF). In a chip experiment comparing transcript levels of slowC cells (frameshift mutant, lacking BRF1 expression) and slowC revertant cell lines (Stoecklin *et al*., 2000), elevated levels of VEGF message were found in the slowC cells (I. Raineri and C. Moroni, in collaboration with U. Certa *et al.* Roche, Basel, unpublished data). This data indicate

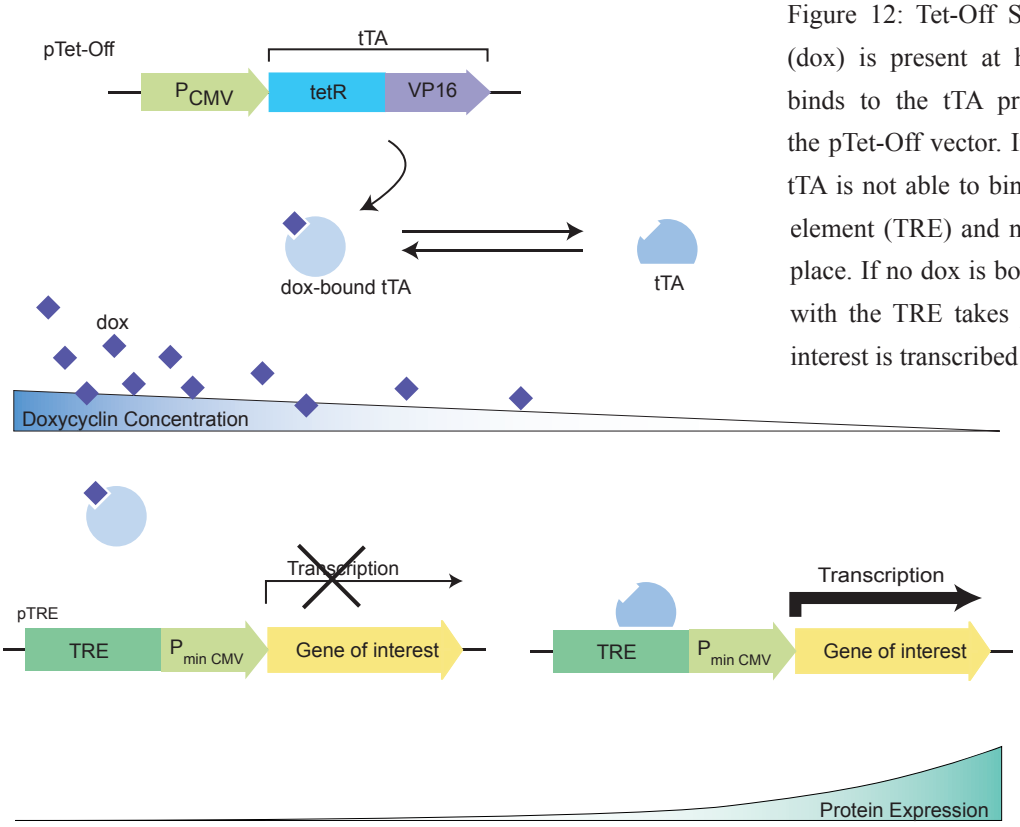


Figure 12: Tet-Off System. If doxycycline (dox) is present at high concentrations, it binds to the tTA protein, expressed from the pTet-Off vector. In the dox bound form, tTA is not able to bind to the tet responsive element (TRE) and no transactivation takes place. If no dox is bound to tTA, interaction with the TRE takes place and the gene of interest is transcribed due to transactivation.

that VEGF message is regulated by BRF1. VEGF is a key regulator of physiological angiogenesis during embryogenesis and skeletal growth. It has been found to induce lymph angiogenesis and also seems to be important in pathological angiogenesis. VEGF is a survival factor for endothelial cells and prevents serum starvation induced apoptosis, probably via the PI3-K-PKB pathway. Expression of this factor is induced by hypoxia and several growth factors (e.g. TGFα, β, FGF, PDGF) (see (Ferrara *et al*., 2003) for review on VEGF). Rat VEGF contains 6 AUUUA pentamers and one nonamer in its 3'UTR. To investigate the stability of this message, HIRc-B cells were left untreated, were treated with insulin alone or pretreated with WM. ActD-chase experiments were performed and the mRNA levels were monitored by northern blotting. VEGF message was expressed at very low levels and disappeared quickly after ActD-treatment (Figure 13B lanes 1-4). Treatment with insulin did stabilize the VEGF mRNA slightly (lanes 5-8). Inhibition of PKB by WM did abolish this effect (lanes 9-12). In both experiments (Figure 13A and B), the message was only stabilized in the first hour after insulin-treatment. This correlates with the fact that PKB activation peaks after 15 minutes of insulin-treatment and decreases after 30 minutes as shown in the Western blot in Figure 13C, where phospho-S473-PKB levels were analyzed under the same conditions. During the writing of this work, it has been reported by Ciais *et al.* that BRF1 interacts with the ARE of VEGF and destabilizes VEGF mRNA (Ciais *et al*., 2004).

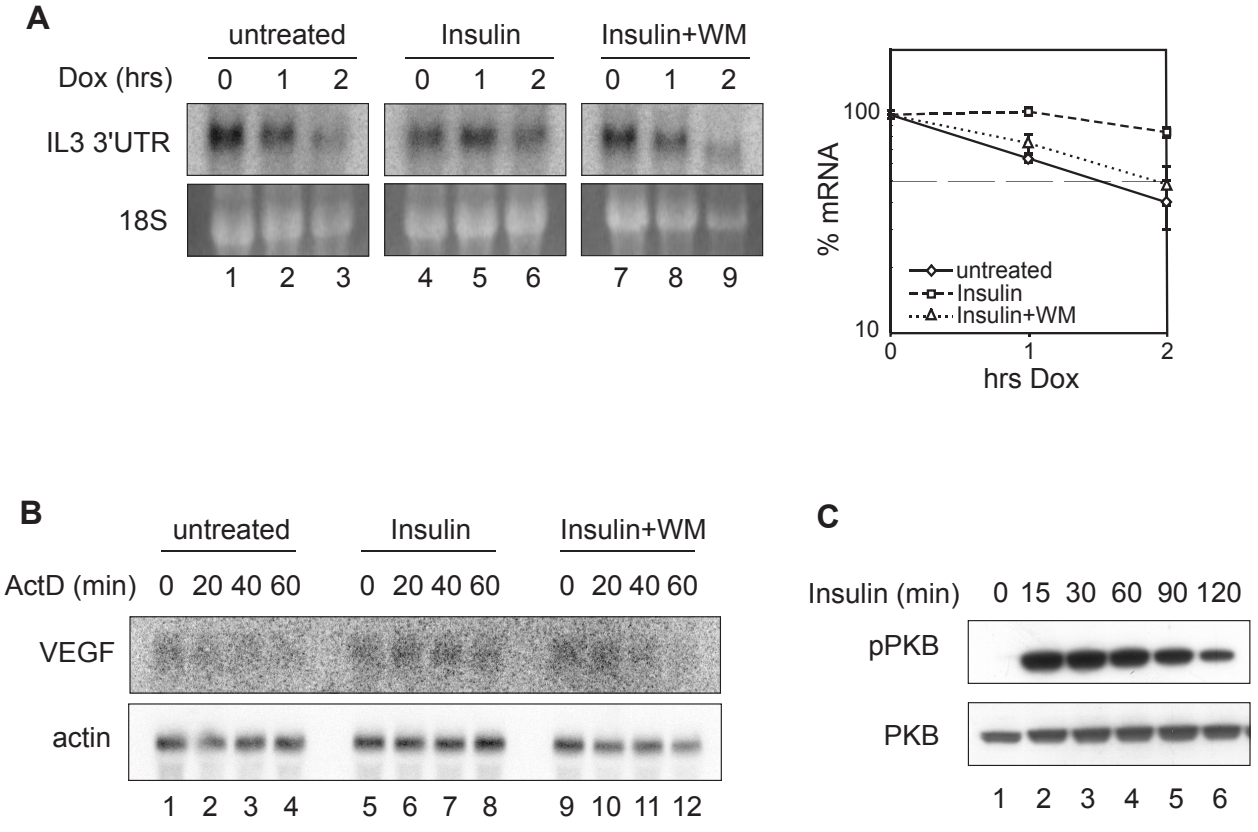


Figure 13 **A:** Dox-repressible β-globin-IL3-3'UTR reporter (pSRL) was cotransfected with the tTA transactivator (pTet-Off) into HIRc-B cells. 24h after transfection, cells were split into 3 dishes. 24h late, cells were treated with WM (200nM) for 30min and/or insulin (20µg/ml) for 15 additional minutes as indicated. Then, cytoplasmic RNA was extracted and reporter mRNA levels were analyzed by northern blotting. The graph shows the mean values of three independent experiments normalized to 18S rRNA with standard deviation. **B:** ActD-chase experiment of endogenous VEGF message in HIRc-B cells. Cells were pretreated for 30min with WM (200nM) and 15 additional minutes with insulin (20µg/ml) as indicated. One representative blot is shown out of three independent experiments. **C:** Time course of PKB activation in HIRc-B cells upon insulin stimulation (20µg/ml), analyzed by Western blot for PKB activation (pPKB) and total PKB levels.

The ARE-Dependent mRNA-Destabilizing Activity of BRF1 is Regulated by PKB

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The ARE-dependent mRNA-destabilizing activity of BRF1 is regulated by protein kinase B

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Butyrate response factor (BRF1) belongs to the Tis11 family of CCCH zinc-finger proteins, which bind to mRNAs containing an AU-rich element (ARE) in their 3′ untranslated region and promote their deadenylation and rapid degradation. Independent signal transduction pathways have been reported to stabilize ARE-containing transcripts by a process thought to involve phosphorylation of ARE-binding proteins. Here we report that protein kinase B (PKB/Akt) stabilizes ARE transcripts by phosphorylating BRF1 at serine 92 (S92). Recombinant BRF1 promoted *in vitro* decay of ARE-containing mRNA (ARE-mRNA), yet phosphorylation by PKB impaired this activity. S92 phosphorylation of BRF1 did not impair ARE binding, but induced complex formation with the scaffold protein 14-3-3. *In vivo* and *in vitro* data support a model where PKB causes ARE-mRNA stabilization by inactivating BRF1 through binding to 14-3-3.

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Introduction

The stability of mRNA varies considerably among different species of transcripts and is in many cases regulated in a complex fashion in response to external stimuli. Short-lived transcripts carry *cis* elements that appear to regulate the access to the mRNA decay machinery. A major element is the so-called AU-rich element (ARE), which is located in the 3′ untranslated region (3′UTR) of many short-lived transcripts from cytokines, proto-oncogenes, growth factors or cell cycle regulators (Shaw and Kamen, 1986; Chen and Shyu, 1995; Bakheet *et al*, 2001). AREs promote deadenylation and decapping (Gao *et al*, 2001), followed by degradation of the mRNA body (Shyu *et al*, 1991; Xu *et al*, 1997). Mammalian ARE-containing mRNA (ARE-mRNA) is thought to be degraded mainly by the exosome, a multiprotein complex containing 3′–5′ exonucleases and helicases (Chen *et al*, 2001; Mukherjee *et al*, 2002). Stabilization of short-lived ARE-containing transcripts by exogenous signals leads to a rapid accumulation of mRNA, with a consequent increase of protein levels. Physiological examples are found in T-cells activated by immune stimulation (Lindsten *et al*, 1989), mast cells responding to IgE-linked allergens (Wodnar-Filipowicz *et al*, 1989; Wodnar-Filipowicz and Moroni, 1990), or macrophages stimulated by IL-1/TNFα (Huang *et al*, 2000), where increased cytokine production occurs. Deregulated ARE-dependent mRNA turnover can contribute to oncogenic transformation (Schuler and Cole, 1988; Nair *et al*, 1994; Stoecklin *et al*, 2003), inflammation (Carballo *et al*, 1998) and immunopathology (Kontoyiannis *et al*, 1999), underlying the physiological relevance of this process. Also, stress stimuli, including UV exposure (Gorospe *et al*, 1998; Wang *et al*, 2000), heat shock (Laroia *et al*, 1999) and hypoxia (Paulding and Czyzyk-Krzeska, 2000), can lead to stabilization of ARE-mRNA. In addition to mRNA turnover, the ARE has been reported to regulate translation (Kontoyiannis *et al*, 1999; Piecyk *et al*, 2000).

Several signal transduction pathways have been implicated in mRNA decay control. Activation of stress-induced c-jun N-terminal kinase (JNK) (Chen *et al*, 1998; Ming *et al*, 1998), p38 mitogen-activated protein kinase (MAPK) (Dean *et al*, 1999; Winzen *et al*, 1999; Brook *et al*, 2000; Clark *et al*, 2003; Frevel *et al*, 2003), MAPKAP kinase 2 (MK2) (Neininger *et al*, 2002), phosphatidylinositol 3-kinase (PI3-K) (Ming *et al*, 2001) and wnt-β-catenin pathway (Briata *et al*, 2003) has been shown to trigger stabilization of various transcripts.

Several ARE-binding proteins (AUBPs) affecting mRNA turnover have been identified. HuR, a member of the embryonic-lethal abnormal vision (ELAV in *Drosophila melanogaster*) family of RNA-binding proteins (Ma *et al*, 1996), stabilizes ARE-containing transcripts (Fan and Steitz, 1998). AUF1 (hnRNPD) exerts a stabilizing as well as a destabilizing function depending on cell type (Zhang *et al*, 1993; Chen *et al*, 2002) and isoform (Raineri *et al*, 2004). The Tis11 protein family members tristetraprolin (TTP) and butyrate response factors (BRF1 and BRF2) share a highly conserved CCCH tandem zinc-finger (Zn-finger) motif that binds RNA (Lai *et al*, 1999). TTP was initially identified by the Blackshear group as a positive regulator of TNFα mRNA decay (Carballo *et al*, 1998) by studying the autoimmune-like phenotype of TTP knockout (k.o.) mice. Subsequently, it was found to act by targeting ARE-mRNA to the exosome (Chen *et al*, 2001). BRF1 was recently identified by a functional genetic screen aimed at finding genes responsible for ARE-dependent decay (Stoecklin *et al*, 2002). Interestingly, BRF1 has been found to be a circadian gene (Storch *et al*, 2002) and its k.o. is lethal

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at embryonic day 11 (Stumpo *et al*, 2004). Both TTP and BRF1 can stimulate deadenylation (Lai *et al*, 2003). TIA/TIAR are RNA recognition motif (RRM)-containing proteins that regulate translational silencing of TNFα mRNA and recruitment into stress granules (Gueydan *et al*, 1999; Piecyk *et al*, 2000). Lastly, KSRP, a K homology (KH)-motif RNA-binding protein originally described as a neuronal specific splicing enhancer of c-src (Min *et al*, 1997), has been recently demonstrated to be an ARE-mRNA-specific destabilizing factor both *in vitro* and *in vivo* (Gherzi *et al*, 2004). An ARE-binding helicase has been described by Tran *et al* (2004).

AUBPs are obvious candidate targets for signaling pathways regulating mRNA turnover. HuR has been shown to act in concert with p38 in stabilizing IL-3 mRNA (Ming *et al*, 2001), although direct phosphorylation of HuR was not detected. TTP was found to be phosphorylated by p38, which inhibited its ARE-binding activity (Carballo *et al*, 2001; Zhu *et al*, 2001). In addition, the extracellular signal-related kinase 2 (ERK2) (Taylor *et al*, 1995) and MK2 (Mahtani *et al*, 2001) are able to phosphorylate TTP *in vitro*. Phosphorylation of TTP by MK2 leads to 14-3-3 binding, inhibition of TTP activity and exclusion of TTP from stress granules (Johnson *et al*, 2002; Chrestensen *et al*, 2003; Stoecklin *et al*, 2004). 14-3-3 may also interact with BRF1, as suggested by results from a yeast two-hybrid screening (Bustin and McKay, 1999) and from GST-pulldown experiments (Johnson *et al*, 2002).

Considerable progress in the analysis of mRNA turnover has been made by the development of *in vitro* decay systems. As control can be exercised over the choice of reagents to be added/omitted, different aspects of post-transcriptional regulation, including deadenylation, decapping and degradation of ARE-containing RNA, can be studied (Ford *et al*, 1999; Chen *et al*, 2000; Gao *et al*, 2001). *In vitro* AREs destabilize RNA through binding of AUBPs such as KSRP and TTP, which in turn recruit both the exosome and the deadenylase, thus targeting the RNA for exosomal decay (Chen *et al*, 2001; Lai *et al*, 2003; Gherzi *et al*, 2004). *In vitro* systems therefore provide a powerful tool to study the regulation of ARE-dependent decay.

Here we show that BRF1 is able to promote ARE-dependent decay in an *in vitro* system, and that this activity is strongly reduced by phosphorylation of BRF1 by protein kinase B (PKB) at serine 92 (S92). Using a phospho-specific antipeptide antibody, we present evidence that BRF1 phosphorylation at S92 mediates insulin-induced mRNA stabilization *in vivo*. Furthermore, BRF1 phosphorylation was found to provide a docking site to 14-3-3, suggesting that this protein may sequester BRF1 from the cellular decay-promoting machinery.

Results

Activated PKB stabilizes ARE-containing reporter mRNA

Previously, we have shown that activated PI3-K is able to stabilize ARE-containing reporter mRNA upon transfection into NIH 3T3 cells (Ming *et al*, 2001). We tested whether PKB, a downstream effector of PI3-K, mediates this effect. An activated form of PKB (m/pPKB) (Andjelkovic *et al*, 1997) was transfected together with a tetracycline-sensitive β-globin reporter construct containing the ARE of IL-3 (Tet-β-globin-IL3UTR) (Stoecklin *et al*, 2002) into NIH 3T3 B_2A_2-23 mouse fibroblasts (Xu *et al*, 1998). These cells stably express the Tet-responsive transcriptional activator tTA. When transcription of the reporter was inhibited by the addition of tetracycline, rapid degradation of the message with a half-life of less than 1 h was observed, while the reporter remained stable in the presence of activated PKB (Figure 1A and B). Activation of PKB was therefore sufficient to stabilize the ARE reporter transcript.

BRF1 is phosphorylated at S92 in vitro

Inspection of the coding sequence of BRF1 (SwissProt database; accession number Q07352) revealed the presence of two overlapping RXRXXS motifs, including S90 and S92, corresponding to PKB consensus phosphorylation sites (Alessi *et al*, 1996). Interestingly, this PKB recognition motif is highly conserved in human, mouse and *Xenopus leavis* BRF1 (Figure 2A), but it is not present in TTP, although these closely related proteins show considerable homology throughout other regions.

To address the question whether BRF1 is indeed a substrate of PKB, we first performed *in vitro* phosphorylation experiments. Either full-length recombinant BRF1 or three fragments consisting of the N-terminus (aa 3–110), the central Zn-finger domain (aa 111–179) or the C-terminus of BRF1 (aa 180–338) were tested as substrates for PKBβ. Only the full-length rBRF1 and the N-terminal fragment containing S90 and S92 proved to be efficiently phosphorylated (Figure 2B, lanes 5–12).

We used the N-terminal fragment (aa 3–110) of BRF1 to identify the site phosphorylated by PKB via mass spectro-

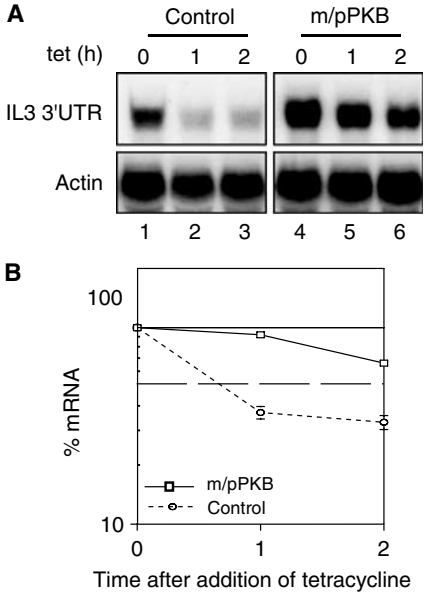

Figure 1 ARE-mRNA stabilization by PKB. (**A**) The Tet-β-globin-IL3UTR reporter gene was transfected alone (lanes 1–3) or in combination with constitutively activated m/pPKB (lanes 4–6). After 24 h, transcription was stopped by addition of doxycycline. Cytoplasmic RNA was isolated at the indicated time points and processed for northern blotting. (**B**) The graph shows the quantifications of five independent decay assays normalized to the actin signal. Standard errors are shown unless too small to be represented.

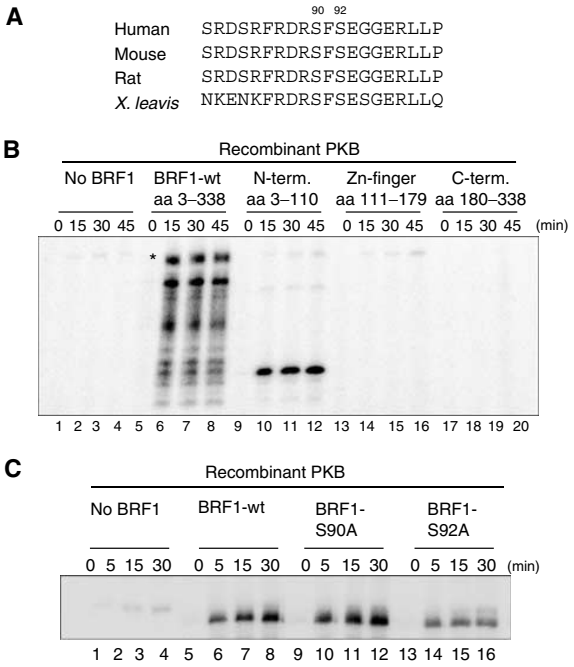

Figure 2 PKB phosphorylates BRF1 on S92. (**A**) Alignment of BRF1 sequences from human, mouse, rat and *X. leavis*. S90 and S92 are indicated. (**B**) *In vitro* phosphorylation reactions were performed for the times indicated using 40 ng of activated recombinant PKB and 20 ng of either full length rBRF1-wt, a N-terminal fragment (aa 3–110), the Zn-finger domain (aa 111–179) or a C-terminal fragment (aa 180–338). The asterisk denotes the full-length BRF1 protein, lower bands are degradation products. (**C**) *In vitro* phosphorylation reactions were performed for the times indicated, using 40 ng of activated recombinant PKB and 20 ng of either rBRF1-wt, rBRF1-S90A or rBRF1-S92A.

metry (see Materials and methods). This revealed that BRF1 is preferentially phosphorylated at S92. Accordingly, a peptide containing the S92 residue (DSRFRDRSFSEG) was phosphorylated *in vitro* by purified activated PKBα and β at 60% of the rate of the GSK3 peptide (RPRTSSFAEG), which is the best-characterized substrate of this kinase (data not shown). To investigate whether S92 is indeed the major PKB phosphorylation site, we mutated S90 or S92 to alanine (S90A, S92A), and tested the recombinant proteins for substrate activity. In line with the mass spectrometry data, BRF1-wt and BRF1-S90A were equally phosphorylated by PKB (Figure 2C, lanes 5–12), whereas phosphorylation was considerably reduced by the S92A mutation (Figure 2C, lanes 13–16). It appears that in the absence of the S92 phosphorylation site PKB can phosphorylate S90, which represents an alternative consensus site.

BRF1 S92 phosphorylation regulates ARE-dependent RNA decay in vitro

We were curious to see whether BRF1 may show similar *in vitro* decay activity as reported for its close homolog TTP (Chen *et al*, 2001), as this would allow us to study the nature of the S92 phosphorylation under controlled *in vitro* conditions. We co-incubated S100 extracts from slowC cells, a line lacking BRF1 expression as the result of frameshift mutations in both alleles (Stoecklin *et al*, 2002), with a radio-labeled RNA containing the 59 nt long ARE from IL-3 (ARE_{IL3}). While ARE_{IL3} was stable in the presence of slowC extracts (Figure 3A, lanes 1–4), it decayed rapidly when 5 ng or more of rBRF1 was added (Figure 3A, lanes 5–20). As a control for both ARE specificity of the observed decay and equal loading, a 250 nt RNA containing the IL-3 3′UTR lacking the ARE (ΔARE) was added at the onset of the reaction. No degradation of the ΔARE control could be observed under all conditions tested. We concluded that rBRF1 is able to promote rapid RNA decay in this ARE-dependent *in vitro* system.

We next investigated whether phosphorylation of rBRF1 by PKB would affect the *in vitro* decay rate. Unphosphorylated rBRF1-wt was compared to rBRF1-wt pretreated with activated PKB for 30 min (Figure 3B, lanes 5–12). A striking reduction of ARE_{IL3} decay was observed with phospho-rBRF1-wt (p-rBRF1-wt) as compared to unphosphorylated rBRF1-wt. To check whether this effect was due to phosphorylation of BRF1 at S92, we performed the same experiment using the rBRF1-S92A mutant. Again, rBRF1-S92A promoted degradation of ARE_{IL3} (Figure 3B, lanes 13–16), yet the mutant appeared refractory to inhibition by PKB (Figure 3B, lanes 17–20). Quantification (Figure 3B, lower panel) revealed an intermediate decay pattern, which may result from alternative phosphorylation at S90 (see also Figure 2C). From these data, we concluded that, *in vitro*, the decay-promoting activity of BRF1 is inhibited by phosphorylation at S92.

BRF1 is phosphorylated at S92 in vivo

To extend this study to endogenous BRF1, we first raised an antibody directed to a trisdekapeptide containing phosphorylated S92 (FRDRSFpSEGGERL). Affinity-purified antibody recognized rBRF1 phosphorylated by PKB with a high specificity (Figure 4A). As an additional control, we preincubated either the antibody described above or an antibody recognizing the C-terminus of BRF1 (Raineri *et al*, 2004) with the S92-containing phosphopeptide (PP; see above). In the case of the phospho-antibody, the western signal could specifically be competed away, while detection of unphosphorylated BRF1 was not impaired (Figure 4B, compare lanes 3 and 6).

We then examined whether BRF1 becomes phosphorylated at S92 *in vivo*. Insulin, a known activator of PKB (Burgering and Coffer, 1995) was used to stimulate HIRc-B rat fibroblasts, which express the human insulin receptor and are widely used to investigate insulin signaling (McClain *et al*, 1987). To reduce PKB background activity, we starved the cells overnight in serum-free medium. After stimulation with insulin for 15 min, PKB was strongly activated (Figure 4C, lane 2, middle panel) and we monitored BRF1 phosphorylation by western blotting using the p-BRF1 antibody. Phosphorylation of BRF1 at S92 could be detected under conditions of PKB activation (Figure 4C, lane 2, upper panel). BRF1 levels did not change in response to insulin stimulation (data not shown). This phosphorylation was partially inhibited by pretreating the cells with the PI3-K inhibitor wortmannin (WM), which completely abolishes PKB activation (Figure 4C, lanes 2 and 3). As WM treatment was not able to block BRF1 phosphorylation completely, we hypothesize that, in addition to PKB, another unidentified kinase may also phosphorylate BRF1 at S92. Phosphorylation of a single serine by more than one

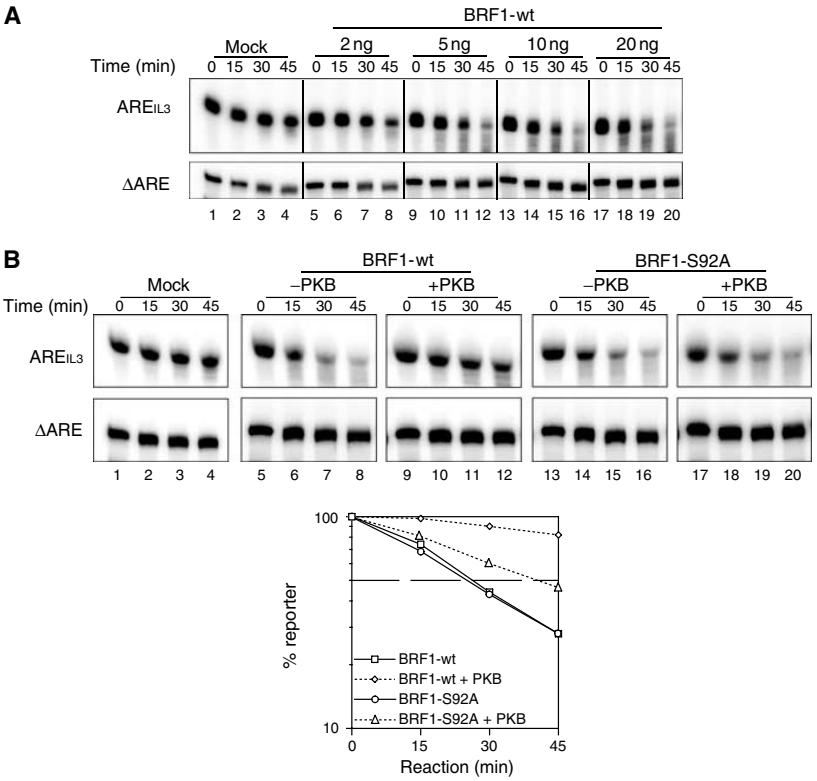

Figure 3 BRF1 S92 phosphorylation regulates ARE-dependent RNA decay *in vitro*. (**A**) Radioactively labeled ARE_{IL3} and ΔARE transcripts were incubated together in S100 extract from slowC cells. Decay reactions were performed at 37 C in the presence of increasing amounts of rBRF1-wt, stopped at the indicated time points and resolved on a 10% urea-polyacrylamide gel. ΔARE transcripts served as specificity and loading control. (**B**) Decay reactions were carried out in the absence (lanes 1–4) or in the presence of 20 ng of rBRF1-wt (lanes 5–12) or rBRF1-S92A (lanes 13–20) after pretreatment with buffer (lanes 5–8/13–16) or activated PKB (lanes 9–12/17–20). The graph below shows quantification of at least four independent experiments after normalizing to the ΔARE signal. Standard error bars were too small to be represented.

kinase is known to occur (Johannessen *et al*, 2004). To obtain *in vivo* evidence that indeed PKB is phosphorylating BRF1, we transfected BRF1-wt or the BRF1-S90A/S92A double mutant construct together with m/pPKB or a kinase-dead variant of PKB (PKBkd) into 3T3 cells. Strong phosphorylation of BRF1-wt, but not of the mutant, was observed in m/pPKB-transfected cells (Figure 4D). Together, these data indicate that PKB does phosphorylate BRF1 *in vivo* at S92.

ARE-mRNA is stabilized under conditions that phosphorylate BRF1

We then wanted to know whether phosphorylation of BRF1 impairs its decay-promoting activity *in vivo*. We co-transfected the Tet-β-globin-IL3UTR reporter together with the tTA transactivator (pTetOff, Clontech) into HIRc-B cells. When transcription of the reporter was turned off by adding doxycycline, we observed rapid decay of the reporter mRNA with a half-life of approximately 1 h (Figure 5A, lanes 1–3). After pretreatment of cells with insulin for 15 min, the mRNA was more stable (lanes 4–6), and this effect was sensitive to WM (lanes 7–9). We concluded that *in vivo* ARE-mRNAs become stabilized under conditions where phosphorylation of BRF1 at S92 occurs. Quantification from three parallel experiments indicated that the observed changes are statistically significant (Figure 5B).

To further evaluate the involvement of PKB in stabilizing ARE-mRNA, we made use of mouse embryonic fibroblasts with k.o. of PKBα (MEF-PKBα$^{-/-}$) (Yang *et al*, 2003). When MEF-PKBα$^{-/-}$ and $+/+$ cells were compared by western blotting, we noticed strong PKB phosphorylation upon insulin stimulation even in $-/-$ cells, apparently via the PKBβ and γ isoforms. In unstimulated MEF-PKBα$+/+$ cells, there was background phosphorylation of BRF1 (Figure 5C), presumably by a kinase other than PKB (see also Figure 4C). Insulin stimulation increased this signal and stabilized the reporter mRNA in a WM-sensitive fashion. In MEF-PKBα cells, the p-BRF1 signal was low with no response to insulin. When mRNA stability of the reporter was examined, decay was generally more rapid in $-/-$ cells and insulin failed to induce stabilization (5D). These data support an *in vivo* role for PKBα in stabilization of ARE-mRNA.

BRF1 phosphorylation leads to 14-3-3 binding

To elucidate the mechanism how PKB inhibits BRF1, we first asked whether BRF1 phosphorylation affects ARE binding, as it was found for TTP (Carballo *et al*, 2001). By electrophoretic mobility shift assay, we compared increasing amounts of unphosphorylated rBRF1 to rBRF1 pretreated with activated PKB for its ability to bind a radio-labeled ARE_{IL3} probe. Phosphorylation did not impair ARE binding

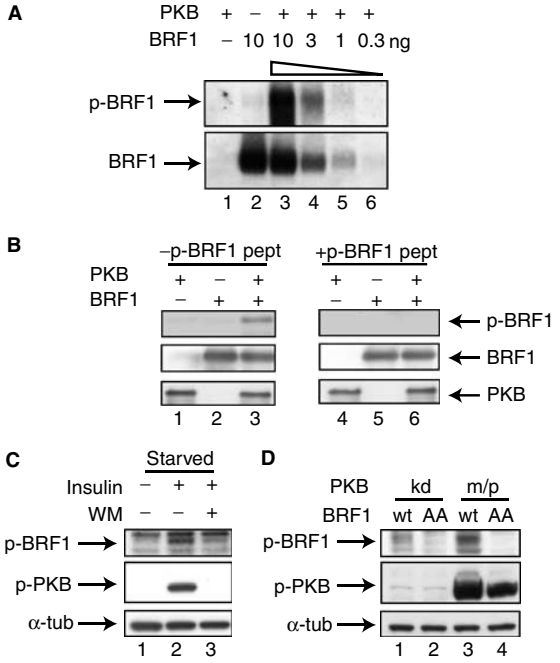

Figure 4 BRF1 is phosphorylated *in vivo* at S92. (**A**) Indicated amounts of rBRF1-wt were incubated with buffer alone or activated PKB for 30′ at 30 C and processed for western blotting. Phosphorylated (upper panel) or unphosphorylated BRF1 (lower panel) were detected by corresponding antibodies directed against a p-S92 containing or a C-terminal peptide (see Materials and methods). (**B**) rBRF1 phosphorylated as in (A) was processed for western blotting. Antibodies recognizing phosphorylated BRF1 (upper panel), BRF1 (middle panel) or PKB (lower panel) were pre-incubated on ice for 30′ with or without 50 μg of the PP used for immunization before adding to the blot. (**C**) HIRc-B cells were serum starved overnight, treated with WM (200 nM) for 30′ where indicated before stimulation with insulin (20 μg/ml) for 15′ (lanes 1–3). Whole-cell extracts were analyzed by western blot, detecting p-BRF1, phospho-PKB and α-tubulin as a loading control. (**D**) NIH3T3 B_2A_2-23 cells were transfected with m/pPKB or kinase-dead PKB (PKBkd) and BRF1-wt or BRF1S90A/S92A (BRF1AA). Whole-cell extracts were analyzed by western blot, detecting p-BRF1, phospho-PKB and α-tubulin as a loading control.

(Figure 6A). The inhibitory effect of S92 phosphorylation on BRF1-destabilizing activity could therefore not be explained by a change in the RNA-binding capacity of BRF1. An alternative mechanism responsible could be binding of phosphorylated BRF1 to an inhibitory factor. A candidate binding partner was 14-3-3, as various isoforms of this protein bind to targets phosphorylated by PKB (Tzivion and Avruch, 2002) and, in addition, 14-3-3 has been found to interact with BRF1 (Bustin and McKay, 1999; Johnson *et al*, 2002). To test this possibility, His-tagged rBRF1, either untreated or phosphorylated by PKB, was added to slowC cell extracts, purified by Ni-NTA beads and processed for western blotting. Phosphorylated but not unphosphorylated BRF1 pulled down 14-3-3 (Figure 6B, lanes 1 and 2). Furthermore, addition of the PP containing phosphorylated S92 antagonized 14-3-3 binding in a dose-dependent fashion (lanes 3–5), whereas the analogous peptide containing the S92A substitution (AP) was unable to interfere with 14-3-3 binding (lanes 6–8).

If interaction with 14-3-3 was responsible for BRF1 inhibition, we would expect the PP to affect binding and hence BRF1 activity in the *in vitro* decay assay. Indeed, addition of PP could restore decay activity of phosphorylated BRF1 (Figure 6C, lanes 9–12), whereas the AP peptide was ineffective (Figure 6C, lanes 13–16). Taken together, these data indicate that PKB-induced inhibition of BRF1 occurs through binding of 14-3-3.

Finally, *in vivo* experiments were performed to verify that PKB regulates the interaction between BRF1 and 14-3-3. For this experiment we chose COS7 cells, as complex formation between phosphorylated TTP and 14-3-3 was recently demonstrated in this line (Stoecklin *et al*, 2004). Cells were transiently transfected with wt or mutant BRF1 constructs carrying a His-tag. BRF1 was purified from the cell extracts using Ni-NTA beads, and western blotting revealed co-precipitation of 14-3-3 with BRF1-wt (Figure 6D, lane 2). Mutation of BRF1 at S90/92 strongly prevented the interaction with 14-3-3 (lane 3). In order to see whether activation of the PKB pathway would stimulate 14-3-3 binding, cells were co-transfected with m/pPKB and serum-starved to reduce endogenous PKB activity. m/pPKB strongly induced binding of 14-3-3 to BRF1-wt (lane 6). Notably, induced binding of 14-3-3 was not observed with the S90A/S92A double mutant (BRF1AA, lane 8). Together, these data suggest that S92 phosphorylation by PKB leads to interaction of 14-3-3 with BRF1. Whereas this interaction does not impair RNA binding, it may inhibit recruitment of the BRF1–RNA complex to the exosomal decay machinery and thereby lead to stabilization of ARE-mRNA.

Discussion

In this report, we provide evidence that phosphorylation of BRF1 by PKB stabilizes ARE-mRNA. As initially reported for PI3-K (Ming *et al*, 2001), its downstream target PKB efficiently stabilized ARE-containing reporter transcripts in transfection experiments (Figure 1). BRF1 contains two overlapping consensus sequences for PKB phosphorylation at S90 and S92, both of which are conserved between mammals and *Xenopus* (Figure 2). Mass spectrometry analysis of a recombinant BRF1 N-terminal peptide phosphorylated by PKB revealed that phosphorylation occurs exclusively at S92. *In vitro* kinase assays with full-length wt protein and S90A/S92A mutants as substrates confirmed S92 as the major phosphorylation site.

To explore the role of S92 phosphorylation, we took advantage of the fact that rBRF1, as previously reported for TTP, is able to promote degradation of ARE-RNA when added to an S100 extract. Interestingly, the decay-promoting activity of BRF1 (Figure 3), but not its ARE-binding activity (Figure 6A), was abrogated by PKB phosphorylation. The rBRF1-S92A mutant was competent in promoting decay, but insensitive to PKB inhibition, establishing S92 as a regulatory site. To our knowledge, this is the first example where specific phosphorylation was shown to affect ARE-dependent mRNA decay *in vitro*.

To validate these findings *in vivo*, we analyzed endogenous BRF1 phosphorylation using a S92 phospho-specific antibody. In HIRc-B cells expressing the human insulin receptor, treatment with insulin triggered rapid phosphorylation of both PKB and BRF1 (Figure 4). The latter effect was only

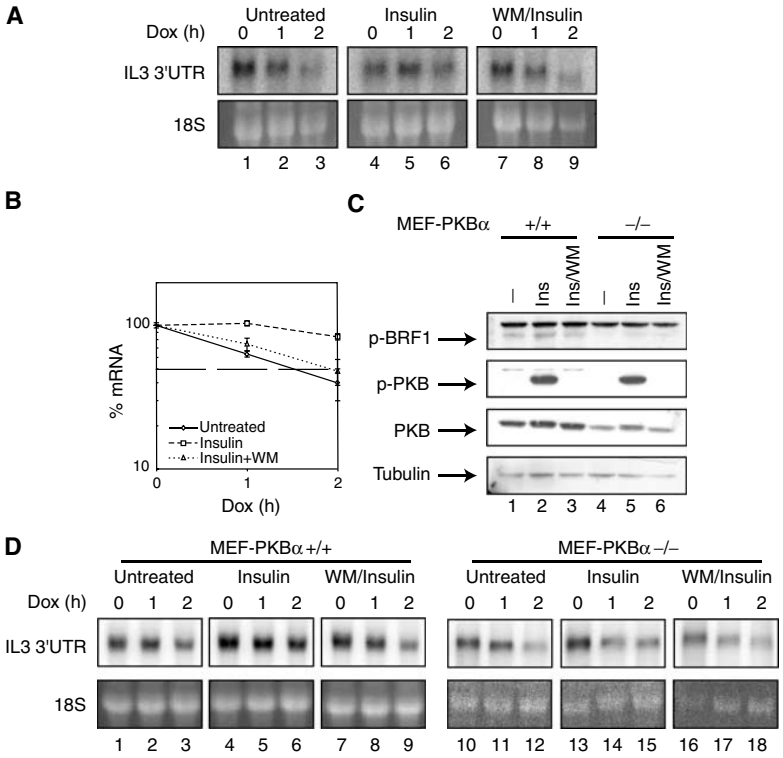

Figure 5 Effect of insulin on reporter mRNA decay. (**A**) At 24 h after co-transfection of the Tet-β-globin-IL3UTR reporter gene and the Tet-responsive transcriptional activator tTA (pTET-on), HIRc-B cells were split into fresh medium for 16 h. Where indicated cells were pretreated with WM (200 nM) for 30′ before addition of insulin (20 μg/ml) for another 15′. Then doxycycline was added (2 μg/ml) to stop transcription, cytoplasmic RNA was isolated at the indicated time points and processed for northern blotting. (**B**) Quantification of three independent experiments is shown normalized to the 18S rRNA signal. (**C**) MEF-PKBα−/− and +/+ cells were pretreated with WM for 30′ prior to insulin (Ins) stimulation for 15′ as indicated. p-BRF1, phospho-PKB, PKB and tubulin protein levels were analyzed by western blot. (**D**) MEF-PKBα−/− and +/+ cells were treated as described for HIRc-B in (A). Reporter RNA was analyzed by northern blot. One representative experiment is shown.

partially blocked by the PI3-K inhibitor WM, suggesting that a second kinase in addition to PKB may also phosphorylate S92. Support for this notion was found in MEF-PKBα+/+ cells, where in the absence of PKB activation background BRF1 phosphorylation was observed (Figure 5C). A single serine can indeed be phosphorylated by more than one kinase, as known from nuclear factor CREB, which becomes phosphorylated at position 133 not only by PKA, but also by PKB and other AGC kinases (Johannessen *et al*, 2004). That PKB can phosphorylate *in vivo* BRF1 at S92 was obvious from transfection experiments, where activated PKB phosphorylated BRF1-wt but not BRF1-S90A/S92A (Figure 4D).

When ARE reporter mRNA decay following insulin stimulation was examined, we observed WM-sensitive stabilization in HIRc-B (Figure 5A) and MEF-PKBα+/+ cells, but not in MEF-PKBα−/− cells. In the latter, the p-BRF1 signal was low under all conditions and correlated with rapid decay.

To explore the consequences of BRF1 phosphorylation, we turned our attention to the 14-3-3 protein. Members of the family of 14-3-3 proteins are known to bind a variety of target proteins phosphorylated by kinases belonging to the AGC kinase family, which includes PKB. The optimal binding site for 14-3-3 is RSXpSXP (Tzivion and Avruch, 2002), with preference for an aromatic or positively charged amino acid at position −1. The sequence surrounding S92 of BRF1 (RSFpSEG, see Figure 2) represents a 14-3-3 binding site only lacking a P at position +2 compared to the optimal consensus. However, BRF1 has been shown to interact with 14-3-3 in yeast two-hybrid assays and pulldown experiments (Bustin and McKay, 1999; Johnson *et al*, 2002). Our experiments demonstrate that PKB induces complex formation between BRF1 and 14-3-3 in the same extracts in which BRF1-dependent *in vitro* RNA decay is regulated by PKB. The S92-containing PP, which competitively dissociated the BRF1/14-3-3 complex, also specifically antagonized the inhibitory effect of PKB on BRF1 activity. As our BRF1 antibody was not able to pull down endogenous protein from HIRc-B cell (data not shown), we overexpressed His-tagged BRF1 in COS7 cells. 14-3-3 co-purified with BRF1-wt, but not with BRF1-S90A/S92A. In addition, co-transfection of activated PKB strongly stimulated binding of 14-3-3 to BRF1-wt, but not to mutant BRF1. Together, our results suggest that BRF1 phosphorylation at S92 leads to binding of 14-3-3, which inhibits the decay-promoting activity of BRF1. Interaction with 14-3-3 has been reported to have diverse functional consequences depending on the targets involved, including inhibition of apoptosis (BAD protein), increasing nuclear export rates (FKH transcription factor), enhancement of DNA binding (p53 protein), protection from proteolysis or by simply serving as a phosphorylation-dependent scaffold

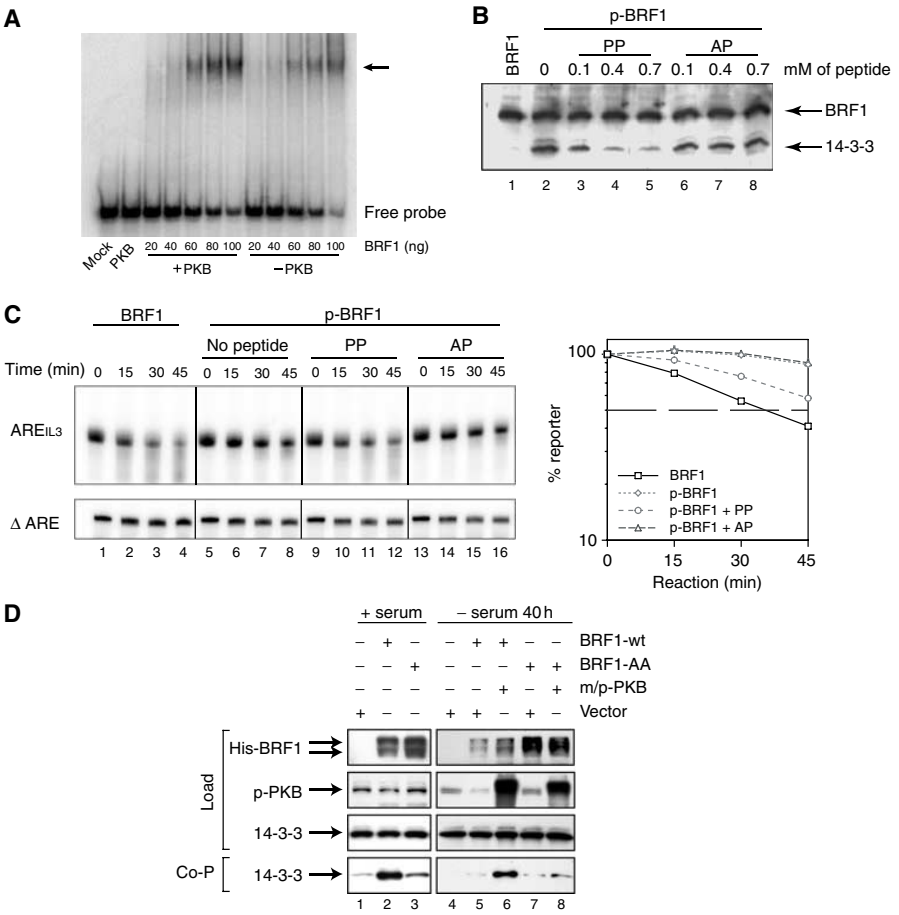

Figure 6 S92 phosphorylation of BRF1 induces complex formation with 14-3-3. (**A**) Electrophoretic mobility shift assay. Indicated amounts of rBRF1 were pre-incubated with or without activated PKB, mixed with radio-labeled ARE_{IL3} RNA and resolved on 4% nondenaturing PAGE. (**B**) His-tagged rBRF1 pretreated with buffer (lane 1) or activated PKB (lanes 2–11) was mixed with S100 extract from slowC cells. Increasing concentrations of the phosphorylated peptide surrounding S92 (aa 86–98) of BRF1 (PP) or the corresponding S92A mutant peptide (AP) were added. rBRF1 was purified using Ni-NTA beads, and 14-3-3 and BRF1 were detected with the corresponding antibodies. The lower band represents 14-3-3, the upper His-tagged rBRF1. (**C**) *In vitro* decay reactions were carried out as described in Figure 3 in the presence of 0.4 mM of PP (lanes 9–12) or AP (lanes 13–16). The graph on the right shows quantification of four independent experiments after normalizing to the ΔARE signal. Standard error bars were too small to be represented. (**D**) COS7 cells were transfected with vector, bsdHisBRF1 wt, bsdHisBRF1 aa, either alone (lanes 1–3) or in combination with m/pPKB (lanes 5–8), as indicated. In the experiment shown on the right (lanes 4–8), FCS was withdrawn for 40 h. Expression of His-tagged BRF1, phospho-PKB and 14-3-3 in the cell extracts was determined by western blotting (load). After purification of BRF1 by Ni-NTA beads, co-precipitation of 14-3-3 was tested by western blotting (Co-P).

(for references, see Tzivion and Avruch, 2002). A reasonable assumption, therefore, is that 14-3-3 sequesters BRF1 *in vivo* and thereby restricts its access to the decay machinery. Interestingly, Stoecklin *et al* (2004) have recently reported that phosphorylation of TTP by MK2 at S178 and S52 leads to formation of a similar complex with 14-3-3, which reduces TTP activity and excludes TTP from the stress granules. While a homologous site to S178 in TTP is present in BRF1 (S201 or 203), the N-terminal S52 is not. On the other hand, TTP does not contain a homolog to S92 in BRF1. It will be interesting to see whether S201 or 203 in BRF1 is also phosphorylated by MK2 or another kinase, as this may allow cooperative regulation of BRF1 by two independent signal pathways. As 14-3-3 proteins generally bind as dimers to two phosphorylated residues on their target, dual phosphorylation of BRF1 may be required for full binding of 14-3-3 and robust inhibition of its activity.

An emerging model of regulated ARE-dependent mRNA decay has the following features: In resting cells, AUBPs such as BRF1, TTP, KSRP or AUF1 form part of a multiprotein complex that binds the ARE and facilitates access of the RNA to the decay machinery. More than one AUBP may act on a given species of mRNA, as shown for KSRP and BRF1 in HT1080 cells (Gherzi *et al*, 2004), and the relative contributions of AUBPs may depend on the cell type. The two Zn-finger domains of the Tis11 gene family proteins recognize the UUAUUUAUU nonamer, the hallmark of class-II AREs, by binding to two adjacent 5′-UAUU-3′ subsites (Hudson *et al*, 2004). Thus, an ARE containing more than one nonamer is likely to bind several AUBPs. Cell activation or stress leads to a change in the phosphorylation status of TTP, BRF1 or AUF1 (Wilson *et al*, 2003a, b), changing their interacting partners and thereby altering the composition of the multiprotein complex targeting the ARE. In such a situation, the stabilizing

HuR protein may be able to bind preferentially to the ARE, while the poly(A)-ribonuclease (PARN), the exosome, the decapping machinery and the 5′ to 3′ exonucleases lose access to the ARE-mRNA. In addition, mRNA has not only the binary choice of being degraded or delivered to the translation machinery, but also may alternatively be stored in stress granules for later translation. Elucidation of the interplay between these complex and dynamic processes requires the identification of the major proteins involved, as well as their post-translational modification and their functional significance.

Materials and methods

Plasmids

The plasmids bsdHisBRF1$_{S90A}$ and bsdHisBRF1$_{S92A}$ were generated from bsd-HisBRF1$_{wt}$ (Stoecklin *et al*, 2002) by site-directed mutagenesis, using as upstream primers TV43 (5′-CCGCGCC TTCTCGGAAGGGGGCGAG-3′) and M2521 (5′-GGAGCGGTCTCGG AAGCGGC-3′), respectively, and as downstream primers TV44 (5′-TCTCGGAAGCGGCTGTCTCGCGAGC-3′) and M2518 (5′-TTCGCC GAAGGGGGCGAGCG-3′), respectively. bsd-HisBRF1$_{S90A}$ served as template to construct bsd-HisBRF1$_{AA}$ using M2518 and M2522 (5′-GGCGCGGTCTCGGAAGCGGC). For plasmid SP6-ARE, a 59 nt fragment spanning the ARE of IL-3, including six AUUUA pentamers, was amplified from murine IL-3 cDNA by PCR using primers M1977 (5′-ATGGATCCTTCCATTAAGGC-3′) and M1978 (5′-ATAGATCTTCACAGAAGGC-3′). The amplicon was digested with *Bam*HI and *Bgl*II, and ligated into the *Bgl*II site of pSP73 (Promega). Plasmids (m/p)-HA-PKBα and T7-ARE^{-} have been described previously (Andjelkovic *et al*, 1997; Stoecklin *et al*, 2002). For recombinant BRF1, the constructs used were pQE-30-BRF1$_{S90A}$, pQE-30-BRF1$_{S92A}$ and pQE-30-BRF1$_{AA}$, which were constructed in the same way as pQE-30-BRF1$_{wt}$ (Stoecklin *et al*, 2002), using bsd-HisBRF1$_{S90A}$, bsd-HisBRF1$_{S92A}$ and bsd-HisBRF1$_{AA}$, respectively. To generate pQE-30-N-term., pQE-30-BRF1$_{wt}$ was digested with *Hin*dIII and *Hin*cII, blunt-ended and religated. For pQE-30-C-term., the *Sap*I (blunt)–*Xho*I fragment of bsd-HisBRF1$_{wt}$ was inserted into the *Sma*I–*Sal*I sites of pQE-30 (Qiagen). For pQE-30-Zn-finger, the *Hin*cII(blunt)–*Sap*I(blunt) fragment of pQE-30-BRF1$_{wt}$ was inserted into the *Sal*I (blunt) site of pQE-30. Recombinant proteins were produced as described (Stoecklin *et al*, 2002).

Cell culture and transfection

SlowC (Stoecklin *et al*, 2002), NIH 3T3 B_2A_2-23 (Xu *et al*, 1998) and COS7 cells were grown in Iscove's modified Dulbecco medium (IMDM) supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HIRc-B cells (McClain *et al*, 1987), MEF-PKBα−/− and +/+ cells (Yang *et al*, 2003) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with FCS, 2-mercaptoethanol, glutamine, penicillin and streptomycin as described above. To reduce the background PKB activity, cells were starved overnight in serum-free medium before stimulation with insulin (20 μg/ml). Transfection was performed using Lipofectamine 2000 reagent (Life Technologies) following the manufacturer's protocol.

In vitro phosphorylation

The reaction was performed in 30 mM Tris–HCl (pH 7.5), 5mM $MgCl_2$, 1 mM DTT, 0.2 mM ATPγS (unlabeled or ^{32}P-labeled) with 4 ng/μl rBRF1 and 10 ng/μl activated PKB for 30′ at 30 C (Yang *et al*, 2002).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as described (Stoecklin *et al*, 2002), with the exception that ARE$_{IL3}$ RNA transcribed from SP6-ARE was used as probe.

ARE-mRNA decay analysis

In vivo measurement of RNA decay in NIH 3T3 B_2A_2-23 and HIRc-B cells using the tetracycline-sensitive Tet-β-globin-IL3UTR reporter gene was performed as described previously (Stoecklin *et al*, 2002). For *in vitro* analysis of RNA decay, S100 fractions were prepared from slowC cells and RNA decay reactions were performed as described previously (Chen *et al*, 2001), with the addition of 100 nM of okadaic acid. ^{32}P-labeled transcripts from SP6-ARE and T7-ARE^{-} (Stoecklin *et al*, 2002) were synthesized *in vitro* using SP6 or T7 RNA polymerase (Promega), respectively. Both transcripts were co-incubated in all reactions.

Northern blotting analysis

RNA extraction and northern blotting analysis have been described previously (Stoecklin *et al*, 2000).

Western blot analysis

The following antibodies were used for Western blotting: sc-629 rabbit anti 14-3-3β (Santa Cruz), sc-7270 mouse monoclonal anti-omni probe (Santa Cruz), phospho-Akt (Ser 473) 587F11 Monoclonal Antibody (Cell Signaling Technology), rabbit polyclonal anti-AKT (Cell Signaling) and mouse monoclonal anti α-tubulin (236-10501) (Molecular Probes).

Anti-p-BRF1 and anti-BRF1 antibodies were generated by immunizing rabbits with the KLH-linked peptides F-R-D-R-S-F-S(PO_3H_2)-E-G-G-E-R-L (aa 86–98) and S-D-Q-E-G-Y-L-S-S-S-S-S-H-S-G-S-D-S-P-T (aa 300–320) (Neosystem). Sera were affinity-purified using the above-mentioned peptides linked to activated CH Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Proteins were visualized using ECL Advance (Amersham) or CDP-star (Roche).

Pulldown assay

In vivo: At 24–48 h after transfection of COS7 cells using lipofectamine 2000, cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 10% glycerol, 20 mM 2-mercaptoethanol, 10 mM imidazole, 1 mM Na-vanadate, 50 mM NaF, 20 nM okadaic acid and EDTA-free 'complete' protease inhibitors (Roche). After incubation of the cytoplasmic fraction for 1 h at 4 C with 40 μl of Ni-NTA magnetic agarose beads (Qiagen), the beads were washed four times in lysis buffer containing 20 mM imidazole, eluted in SDS-sample buffer and resolved on 4–20% gradient polyacrylamide tris-glycine gels (Invitrogen).

In vitro: In all, 40 ng of phosphorylated or unphosphorylated rBRF1 and different amounts of the peptides PP (F-R-D-R-S-F-S(PO_3H_2)-E-G-G-E-R-L corresponding to aa 86–98) or AP (F-R-D-R-S-F-A-E-G-G-E-R-L; Neosystem) were incubated with 40 μg of S100 slowC extract in 100 mM Tris–HCl (pH 8.0), 1 mM Mg-acetate, 1.5 mM K-acetate, 150 mM NaCl, 10% glycerol, 10 mM imidazole and 100 nM okadaic acid in a total volume of 800 μl. After incubation at 37 C for 30 min, Ni-NTA affinity purification was performed as described above.

Mass spectrometry

The N-terminal BRF1 band (aa 3–110) was excised from the gel, reduced with 10 mM DDT, alkylated with 55 mM iodoacetamide and cleaved with 0.5 μg LysC (Achromobacter, Wako BioProducts) at 37 C overnight in 25 mM ammonium bicarbonate buffer (pH 8.0). After the LysC cleavage, the gel was dried and 0.5 μg of Asp-N (Roche; sequencing grade) was added in 40 μl of 50 mM sodium phosphate buffer (pH 8.0) at 37 C for 6 h. The extracted peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MSMS) using a Magic C18 100 μm × 10 cm HPLC column (Spectronex) connected on-line to an iontrap Finnigan DecaXP (ThermoFinnigan). PPs were identified with the neutral loss function, searching for fragment ions formed by the loss of phosphoric acid, 32.3, 48.5 or 97 Da from the $(M+3H)3+$, $(M+2H)2+$ and $(M+H)+$ ions, respectively. The PP was isolated by LC-MSMS, followed by cleavage with 0.1 μg trypsin (Promega; sequencing grade) in 50 mM ammonium bicarbonate (pH 8.0) at 37 C for 1 h. The peptide mixture was then analyzed by LC-MSMS as described above and the PP was additionally sequenced by an MS3 experiment.

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WM-Insensitive Kinase Phosphorylates BRF1 at S92

As seen in Figures 10B and 11B, in addition to PKB, a WM-insensitive kinase phosphorylates BRF1 at S92 in insulin stimulated HIRc-B cells. To further analyze the nature of this WM-insensitive phosphorylation, cells were starved overnight, pretreated with inhibitors for MEK1/2 (UO126), p38 MAPK (PD169316) and PI3-K (WM) for 1h, prior to stimulation with insulin. Then, cellular extracts were processed for Western blotting. As a control for the effect of the inhibitors, activated PKB (pPKB, control for WM), activated p38 (pp38, control for PD169316) and activated ERK1/2 (pERK1/2, control for UO126) were detected with the respective antibodies. In the HIRc-B cells, insulin did activate all three tested kinases (Figure 14A, lane 2, panels pPKB, pp38, pERK1/2). Looking at the phosph-S92-BRF1 (topmost panel), again three bands were detected, the highest being unspecific and serving as loading control and the middle one being the band of interest. This middle band is absent in starved cells (lane 1) and induced by insulin (lane 2). In this experiment, WM could only marginally inhibit this phosphorylation (lane 3). Also the p38 inhibitor PD169316 could not reduce the S92 phosphorylation (lane 4; total protein levels are slightly reduced in this lane). Interestingly, UO126 did partly reduce S92 phosphorylation (compare lane 2 with 5, 7, 8

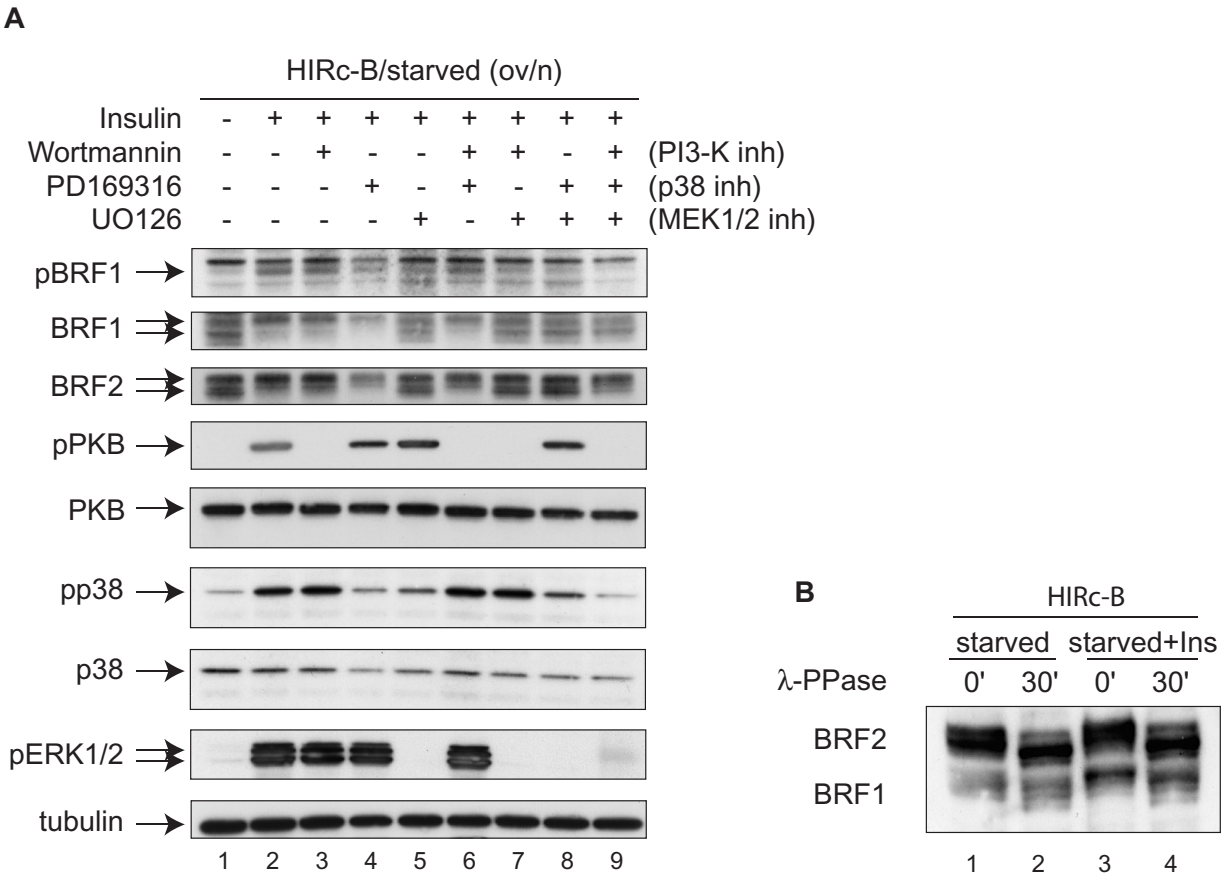


Figure 14: **A:** HIRc-B cells were starved overnight, pre-treated with inhibitors for PI3-K (WM), p38 (PD169316) or MEK1/2 (UO126) before insulin stimulation (15min, 20µg/ml). Protein extracts were processed for Western blot and BRF1 phosphorylated at S92 (pBRF1), total BRF1, BRF2, activated PKB (pPKB), total PKB, activated p38 (pp38), total p38 activated ERK1/2 (pERK1/2) and tubulin. **B:** Western blot of endogenous BRF1 and BRF2 from starved or insulin (15min, 20µg/ml) treated HIRc-B cells. The cells were lysed in absence of phosphatase inhibitors and incubated for 30min with λ-PPase where indicated.

and 9, upper panel). Combination of all three inhibitors completely abolished detectable BRF1 S92 phosphorylation (lane 9). These data argue that S92 phosphorylation upon insulin in HIRc-B cells is not only mediated by PKB, but most likely also by ERK1 or 2, the two downstream kinases of MEK1/2.

BRF1 is Phosphorylated at Additional Sites to S92

Apart from the two PKB consensus sites, many additional putative phosphorylation motifs can be found in the sequences shown in Figure 8. Serine 54 (S54) is a putative PKA site (R-(X)-X-S). According to Stokoe *et al.* (Stokoe *et al*., 1993), S54 matches also to the MK2 consensus (ϕ–X-R-X-X-S). As discussed previously, S90 and 92 are placed in the context of two overlapping PKB consensus sites (R-X-R-X-X-S-ϕ) (Obata *et al.*, 2000), which closely resemble the PKA consensus (Obenauer *et al.*, 2003). S203 is the homologous site to S178 in TTP, which is phosphorylated by MK2 (Chrestensen *et al.*, 2003; Stoecklin *et al.*, 2004) and is also a putative PKC site (Obenauer *et al.*, 2003). Further, we find an ERK1 site at threonine 227 (T227) (P-(X)-X-S/T-P) (Davis, 1993), an MK2 site (S283) and a GSK3 site (S294) (Davis, 1993; Pearson and Kemp, 1991). The phosphorylation consensus sequences for these different kinases are very similar and they vary slightly between different references. Therefore, this sequence searches may only serve as a lead for further investigations.

Apart from the sequence analysis, there is also experimental evidence for additional phosphorylation sites to S92: BRF1 (endogenous and exogenous) can be detected as multiple, differently migrating bands by Western blot. In addition, and in contrast to the *in vitro* decay data (Schmidlin *et al.*, 2004), activated PKB is not able to fully inactivate the decay promoting activity of cotransfected BRF1 in ActD-chase experiments. Activated rasV12, however, is completely dominant over the destabilizing activity of cotransfected BRF1wt and BRF1S90/92A (Sabrina Leuenberger, PhD thesis, 2004 and unpublished data by Min Lu). This argues that in addition to the PKB pathway, targeting S92, also other pathways, phosphorylating different sites, are important for BRF1 regulation

Insulin-Induced Phosphorylation

Analyzing endogenous BRF1 in the experiment shown in Figure 14A (second panel from top), an interesting pattern can be observed. In HIRc-B cells, the multiple BRF1 bands, observed under unstimulated conditions (lane 1), condense to one slowly migrating, probably hyperphosphorylated form after insulin-treatment (lane 2). This insulin-induced upshift is WM (lane 3) and PD169316 (lane 4) insensitive. Pre-treatment of HIRc-B cells with the MEK1/2 inhibitor UO126, on the other hand, did reverse this insulin effect (compare lane 2 with lanes 5, 7, 8 and 9). The insulin-induced upshift was only observed in the HIRc-B but not in NIH3T3 cells (Figure 11A, lane 2). Overexpression of the human insulin receptor in HIRc-B cells renders this cell line hyper sensitive to insulin, activating also signaling pathways different from the PKB-pathway. Activation of ERK1/2 (pERK1/2) and p38 MAPK (pp38) upon insulin-stimulation is shown in lane 2. As the BRF1 antibody also recognizes BRF2, the phosphorylation pattern of BRF2 could also be investigated. Interestingly, BRF2 behaved completely the same way, as BRF1 (compare second and third panel

from top). In the presence of the UO126, the BRF2 pattern resembles the one from unstimulated cells, whereas the other inhibitors had no effect on the insulin-induced hyperphosphorylation. To check, whether the observed upshift of BRF1 and BRF2 is, indeed, due to phosphorylation, cells were lysed in buffer lacking phosphatase inhibitors and the extract was treated with Lambda-Proteinphosphatase (λ-PPase) at 30°C for 30 minutes. As shown in Figure 14B, phosphatase-treatment did not only reduce the insulin-induced upshift of the two proteins (lanes 3 and 4), but also condensed the multiple bands in unstimulated extracts to a faster migrating form. Therefore, the different forms of BRF1 arise from phosphorylation.
Taken together, Figure 14 shows that BRF1 is phosphorylated in response to insulin at S92. In addition, an insulin-induced shift can be observed on Western blot. The phosphorylation(s) responsible for this upshift seem to be mediated by the ERK1/2 pathway, as the MEK1/2 inhibitor UO126 inhibits this phosphorylation. The fact thet transfected BRF1S90/92A runs similar to the wild-type protein (Figure 10B) indicates that the site responsible for the different BRF1 forms is not S92.

Arsenite-Induced Phosphorylation

Arsenic compounds produce a variety of stress responses in mammalian cells. Arsenite (arsenic trioxide) stress, at the molecular level, shares many features with the heat shock response. Similar to heat stress, arsenite induces heat shock proteins (HSPs) of various sizes and activates ERK, JNK and p38. Through the JNK and p38 pathways, arsenite activates the immediate early genes *c-fos* and *c-jun*. Like other oxygen radical-producing drugs, arsenite induces nitric oxide production, nicotinamide adenine dinucleotide (NAD) depletion, DNA strand breaks, and formation of micronuclei. (Bernstam and Nriagu, 2000; Liu *et al*., 1996) Further, arsenite has been found to induce stress granules, subcellular compartments, where translationally silent mRNAs are stored (Kedersha and Anderson, 2002).
As arsenite-treatment activates many different pathways, this drug might induce BRF1 phosphorylation and if so, provide a tool to investigate the pathways involved. To test this, NIH3T3 cells were transfected with myc-BRF1wt and were treated with arsenite (1mM for 1h). As seen in Figure 15A, two major bands of BRF1 were detected in untreated cells (Figure 15A, lanes 1 and 3, upper panel). After treatment with arsenite, the multiple bands of BRF1 were condensed into one high migrating form. (Figure 15A, compare lanes 1 and 2). This effect was WM-insensitive (lanes 3 and 4), indicating that the PI3-K-PKB axis plays no role. If so, phosphorylation of S92 should not be involved in this upshift. To verify this, the influence of arsenite on the BRF1S90/92A mutant was investigated. Indeed, multiple bands were detected for myc-BRF1S90/92A in untreated cells (Figure 15A, lane 5) and arsenite-treatment did alter the migration of mutant BRF1 similar to the wild-type (Figure 15A, lane 6). This effect was again WM-insensitive (lanes 7, 8). These data show that arsenite-treatment induces modification of BRF1, most likely phosphorylation, which does not involve S90/S92 and which is not mediated by the PI3-K-PKB pathway. It still remained to be confirmed that the observed change in migration is due to phosphorylation. To address this question, cell lysates were again treated with λ-PPase. Extracts from untreated cells (Figure 15B, lane 2) or from arsenite-treated cells (lanes 3-5) were incubated with 100units of λ-PPase for the indicated amount of time. As a control, 100units λ-PPase without extract were loaded in lane 1.

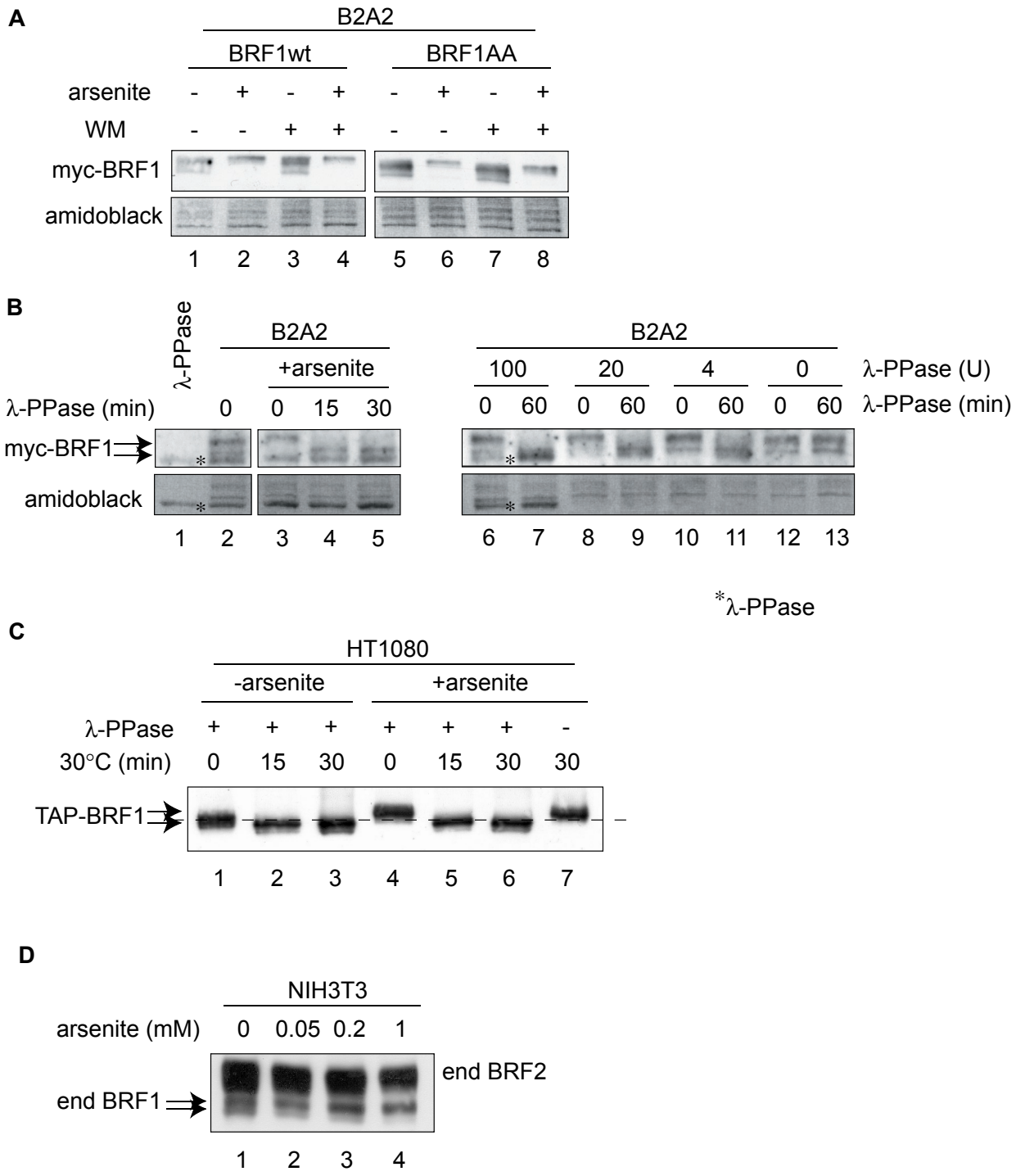


Figure 15: **A:** pTRE-BRF1 (BRF1wt) and pTRE-BRF1S90/92A (BRF1AA) were transfected into NIH3T3 cells (B2A2 clone). 24h after transfection, cells were stimulated with arsenite alone (1mM/ 1h) or pretreated with WM (200nM) for 30min as indicated. Immunoblotting against the myc-tag was performed. **B:** Cell extracts of pTRE-BRF1 transfected NIH3T3 cells (B2A2 clone) were treated for different time periods (lanes 1-5) or with different amounts of Lambda-Proteinphosphatase (λ-PPase) (lanes 6-13) as indicated before processing for Western blot. (The asterisk denotes the band arising from the λ-PPase) **C:** TAP-tagged BRF1 was expressed in HT1080 cells. Its electrophoretic mobility was investigated in response to arsenite (1mM/ 1h). Extracts were treated for indicated time periods with λ-PPase (20units). **D:** Endogenous BRF1 and BRF2 in NIH3T3 cells were analyzed by Western blot. Cells were stimulated with arsenite for 1h, as indicated.

The Western signal was visualized using CDP-star® (Roche), which is activated by phosphatases. Therefore, a background signal, arising from the λ-PPase, was detected (denoted with an asterisk). Again, arsenite-treatment before lysis induced an upshift of BRF1 (compare lanes 2 and 3). The slow migrating form of BRF1 shifted to a single fast migrating form already after 15 minutes of phosphatase-treatment (lanes 3-5). This backshift was dependent on the λ-PPase. Lanes 6 to 13 of Figure 15B show myc-BRF1 in unstimulated NIH3T3. The extracts were treated with decreasing amounts of λ-PPase as indicated. Reduction of the amount of λ-PPase reduced the backshift observed (lanes 6 to 11). Incubation of the extracts at 30°C in absence of λ-PPase did not alter the migration of BRF1 after 60 minutes (lanes 12 and 13).

To assure that BRF1 and not the myc-tag is phosphorylated, the same set of experiments were performed using tandem-affinity-purification (TAP)-tagged (provided by Don Benjamin; Figure 15C) and endogenous BRF1 (Figure 15D). The TAP-BRF1 fusion protein was expressed in HT1080 cells and showed a different pattern from the myc-tagged version, which arose from the different sizes of these two proteins (the TAP-tag adds approximately 20 kDa to the protein size). Only one major band was detected. However, arsenite induced an upshift of this band (Figure 15C, compare lane 1 with lanes 4 and 7). λ-PPase-treatment markedly increased the electrophoretic mobility of TAP-BRF1 in both untreated (lanes 1-3) and arsenite-treated cells (lanes 4-6).

Endogenous BRF1 (Figure 15D) was also analyzed. Treatment of NIH3T3 cells with increasing amounts of arsenite induces an upshift of BRF1 (lanes 1 to 4, with the upper arrow showing the hyperphosphorylated form).

Taken together, it can be stated that BRF1 exists as differentially phosphorylated forms in exponentially growing NIH3T3 cells. Upon arsenite stimulation BRF1 becomes hyperphosphorylated in a WM-insensitive manner.

To address the question, which pathways are involved in the arsenite-response, NIH3T3 cells were pretreated for 1h with inhibitors for MEK1/2 (UO126 and PD98059), p38 (SB202190 and PD169316) and PKB (SH-5) before activation with arsenite or TPA, a strong activator of PKC. Only one inhibitor is shown per kinase, as the corresponding inhibitors had an identical effect. As expected, the PKB inhibitor did not alter the migration of BRF1 (data not shown). It has already

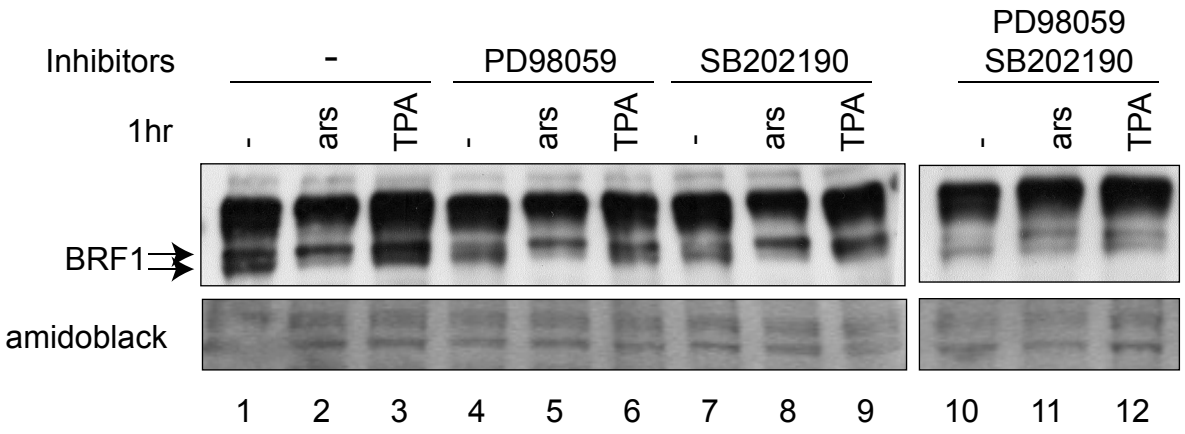


Figure 16: NIH3T3 cells were pretreated for 1h with inhibitors for MEK1/2 (PD98059, 25μM) or p38 (SB202190, 2,5μM) before stimulation with arsenite (1mM) or TPA (200nM) for another hour. The electrophoretic mobility of endogenousBRF1 was investigated by Western blot. As a loading control, amidoblack staining of the membrane is shown.

been discussed above, that PKB mediated BRF1 phosphorylation is not visible as a shift on a SDS-PAGE (Figures 10B and 15A). The MEK and the p38 inhibitors alone slightly reduced the high migrating form of endogenous BRF1 in the unstimulated extracts (Figure 16, compare lane 1 with lanes 4 and 7); however, the arsenite-induced hyperphosphorylation could not be inhibited (compare lane 2 with lanes 5, 8). TPA-treatment did not alter the pattern of BRF1 (lane 3), but, in contrast to unstimulated extracts (lanes 1, 4, 7), the presence of the inhibitors did not reduce the high migrating form of BRF1 in TPA-treated cells (lanes 6, 9). Interestingly, combination of the MEK and the p38 inhibitor did result in a more prominent effect: In unstimulated extracts only the fast migrating (unphosphorylated) form of BRF1 was detected (lane 10), whereas in arsenite-treated cells, in addition to the hyperphosphorylated form, a faster migrating band appeared (lane 11). Only the TPA-treated extracts did not respond to the inhibition. The fact that phosphorylation of BRF1 was reduced in unstimulated cells by the combination of the two inhibitors, but not, if the cells were treated with TPA, indicates that TPA also can induce BRF1 phosphorylation. Together these data suggest that arsenite-treatment leads to phosphorylation of BRF1 by different kinases, probably from the p38 and the ERK pathway, as only combination of both inhibitors was able to reduce the arsenite-induced hyperphosphorylation. It is also possible that the two kinases target the same site. In addition, probably also PKC, which is strongly activated by TPA and not responding to the MEK and p38 inhibitors, phosphorylates BRF1.

Lipopolysaccharide (LPS)-Treatment does not Affect the Motility of BRF1

LPS is a major component of the outer membrane of Gram-negative bacteria, and a very potent activator of microbial inflammation. LPS activates monocytes and macrophages to produce pro-inflammatory cytokines, such as TNFα, IL-1, IL-6, IL-8 and IL-12. LPS-induced macrophage activation involves various kinase pathways including PKA, PKC, Src–related kinases and the MAPK ERK1/2, p38 and JNK (see (Fujihara *et al*., 2003) and references therein). Treatment of RAW 264.7 macrophages has been reported to induce TTP expression and binding of TTP to TNFα mRNA, dependent on the p38 MAPK pathway (Mahtani *et al*., 2001). In the same report multiple TTP phosphorylation has been reported in response to LPS. Therefore, the question was asked, whether LPS also induces BRF1 phosphorylation. RAW 264.7 macrophages were stimulated by LPS and cells were lysed at different time points. Western blotting showed that BRF1 is not expressed at detectable levels in unstimulated macrophages (Figure 17, lane 1, 5). Upon LPS-treatment BRF1 expression

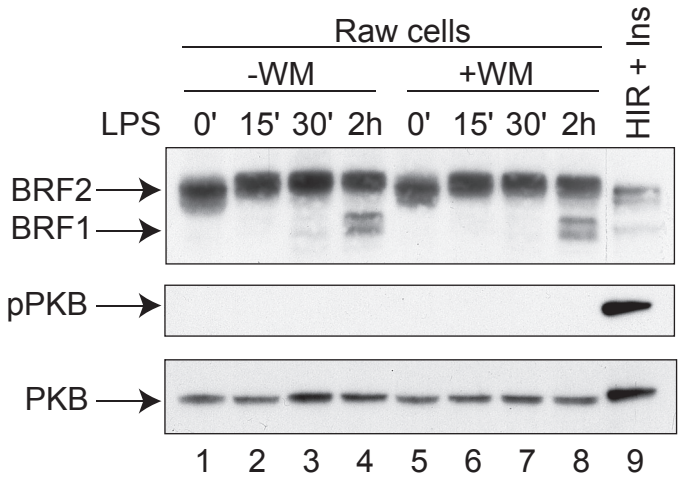


Figure 17: Raw 264.7 macrophages were pretreated with WM (200nM/ 1h) before LPS stimulation (5µg/ml) as indicated. BRF2, BRF1, pPKB and PKB protein was analyzed on Western blot.

was induced (lanes 3, 4, 7 and 8), but did not show the hyperphosphorylated pattern observed in the case of arsenite-treated 3T3 cells or insulin-treated HIRc-B cells (Figure 15/ Figure 17, lane 9). Interestingly, BRF2 expression was constantly high and, in addition, already after 15min BRF2 shifted to a higher migrating form on the SDS-PAGE. This upshift was WM-insensitive. Phosphorylation of BRF1 and BRF2 was very similar in the case of arsenite or insulin-treatment, but the behavior of these two proteins differs in response to LPS-treatment. Dependent on the cell-type and the stimulus the two proteins seem to be affected by the same or by different kinases. In addition, it can be seen in Figure 17 that PKB is not activated by LPS (see middle panel), indicating that the upshift of BRF2 is not dependent on this kinase.

Phosphorylation of BRF1 by Transfection of Activated Kinases

To further narrow down the kinases involved in BRF1 phosphorylation, cotransfection experiments were performed, where activated kinases (m/pPKB, rCD2-p110, MEK6DD, MK2EE, RafCT) and activated rasV12 were transfected together with myc-tagged BRF1wt and the mobility of BRF1 was compared by Western blotting. Activated MEK6 (only slightly), Raf (RafCT) and ras (rasV12) were able to upshift BRF1 (Figure 18, lanes 5, 7 and 8). PKB (m/pPKB) and PI3-K (rCD2-p110) did not change BRF1 migration (lanes 3 and 4). Although p38 seems to be involved in arsenite-induced BRF1 phosphorylation (Fig. 16) and the p38 downstream kinase MK2 has been shown to phosphorylate TTP, transfection of activated MK2 did not alter the migration of BRF1.

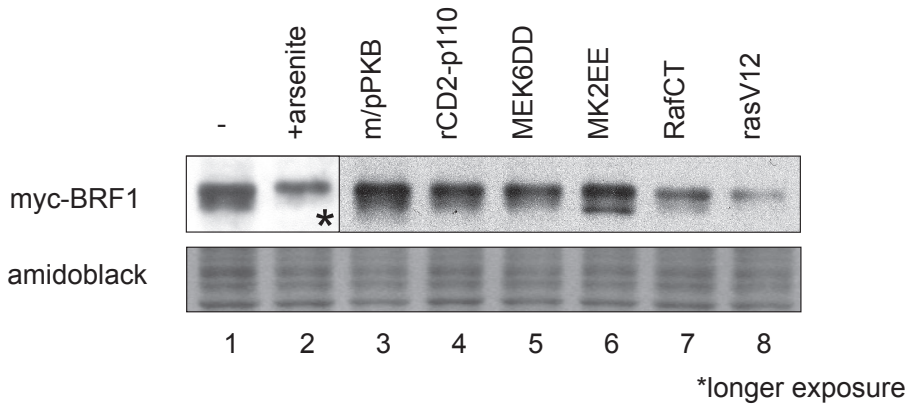


Figure 18: myc-tagged pTRE-BRF1 was transfected together with activated kinases (m/pPKB, rCD2-p110, MEK6DD, MK2EE, RafCT) and rasV12 into NIH3T3 cells (B2A2 clone) and the electrophoretic mobility was analyzed by Western blot. (*Expression of BRF1 was changed upon cotransfection of the different kinases due to effects on the promoter. Therefore, lanes 1 and 2 were exposed longer for equal signal intensity.)

Together, these data support the previous finding: In addition to PKB, also p38 (downstream of MEK6) and ERK (downstream of Raf) are able to phosphorylate BRF1 and PKB mediated phosphorylation does not affect the electrophoretic mobility of BRF1. Thus, this protein seems to be regulated via multiple pathways. Whether p38 mediated phosphorylation is direct or depends on any downstream factors, such as MK2, still remains to be elucidated. Although this set of experiments implicate that MK2 is not involved in BRF1 phosphorylation, it is difficult to compare different kinases in this assay, as the activating mutations not necessarily lead to a comparable degree of activation.

In summary the data presented so far show that BRF1 is phosphorylated at S92 by PKB (Schmidlin *et al.*, 2004) and, dependent on the cell, also by at least one other kinase, most probably ERK1 or 2. In addition, BRF1 is also phosphorylated at site(s) different from S92 by kinase(s) from the p38, the MEK1/2 and/or the PKC pathway. In the case of insulin stimulation in HIRc-B cells, mainly the MEK pathway seems to be involved, whereas in the arsenite-treated NIH3T3 cells also p38 seems to play a role. The functional significance of these diverse phosphorylation events still remains to be elucidated. However it is likely that, *in vivo*, inactivation of BRF1 requires more than one signal.

Mutational Analysis of Putative MK2 Sites

The putative phosphorylation sites (see Figure 8) were now studied in more detail. Publications by Chrestensen, Stoecklin and coworkers (Chrestensen *et al.*, 2003; Stoecklin *et al.*, 2004) proposed a role for MK2 in TTP phosphorylation at serines 54 and 178. This phosphorylation inactivates TTP, probably, through binding to 14-3-3. Comparison of the TTP and the BRF1 protein sequence revealed S203 in BRF1 as the homologues site of one of the two MK2 phosphorylation sites in TTP (S178) (Figure 19). All the putative phospho-sites of BRF1 are conserved in BRF2, whereas none of these sites, except S203, can be found in TTP. This implies that the three proteins despite their homology are differently regulated. Further, S203 and its neighboring amino acids seem to be a common motif of all three mammalian Tis11

```
        1                                                   50
BRF1    MTTTLVSATI FDLSEVLCKG NKML.NYSAP SAGGCLLDRK AVGTPAGG..
BRF2    MSTTLLSA.F YDV.DFLCKT EKSLANLNLN N....MLDKK AVGTPVAAAP
TTP     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        51          S54                                     100
BRF1    ......GFPR RHSVT..... ...LP..... SSKF....HQ NQLLSS...L
BRF2    SSGFAPGFLR RHSASNLHAL AHPAPSPGSC SPKFPGAANG SSCGSA...A
TTP     ~~~~~~~~~~ ~~~~~~~MDL TAIYESLLSL SPDVPVPSDH GGTESSPGWG

        101                                  S92            150
BRF1    KGEP...... .......APA LSSRDSRF.. .RDRSFSEGG ER...LLP..
BRF2    AGGPTSYGTL KEPSGGGGTA LLNKENKF.. .RDRSFSENG DRSQHLLH..
TTP     SSGPWSLSPS DSSPSGVTSR LPGRSTSLVE GRSCGWVPPP PGFAPLAPRL

        151                                                 200
BRF1    ...TQKQP.. .GGGQVNSSR YKTELCRPFE ENGACKYGDK CQFAHGIHEL
BRF2    ...LQQQQKG GGGSQINSTR YKTELCRPFE ESGTCKYGEK CQFAHGFHEL
TTP     GPELSPSPTS PTATSTTPSR YKTELCRTFS ESGRCRYGAK CQFAHGLGEL

        201                                                 250
BRF1    RSLTRHPKYK TELCRTFHTI GFCPYGPRCH FIHNAEERR. ALAG..ARDL
BRF2    RSLTRHPKYK TELCRTFHTI GFCPYGPRCH FIHNADERRP APSGGASGDL
TTP     RQANRHPKYK TELCHKFYLQ GRCPYGSRCH FIHNPSE... ........DL

        251                       S203                      300
BRF1    SA........ ......DRPR LQHSFSFAGF PSA......A ATAAATGLLD
BRF2    RAFGTRDALH LGFPREPRPK LHHSLSFSGF PSGHH..QPP GGLESPLLLD
TTP     AAPGHP.... ........PV LRQSISFSGL PSGRRTSPPP PGLAGPSLSS

        301    T227                                         350
BRF1    ...SPTSITP PP........ .......... .......... ..........
BRF2    ...SPTSRTP PPPSCSSASS CSSSASSCSS ASAASTPSGA PTCCASAAAA
TTP     SSFSPSSSPP PPGDLPLSPS AFSAAPGTPL ARRDPTPVCC PSCRRATPIS

        351                                                 400
BRF1    ....I..L.. ..SADDLL.. GSP....TLP DGTNNPFAFS SQELA...SL
BRF2    AAAAL..LYG TGGAEDLLAP GAPCAACSSA SCANNAFAF. GPELS...SL
TTP     VWGPLGGLVR TPSVQSLGSD PDEYASSGSS LGGSDSPVFE AGVFAPPQPV

        401                                                 450
BRF1    .......... FA........ .......... .......... PSMGLPGGGS
BRF2    ITPLAIQTHN FAAVAAAAYY RSQQQQQQQG LAPPAQPPAP PSATLPAGAA
TTP     AAPRRLPIFN RISVSE~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        451            S283        S294                     500
BRF1    .....PTTF. LFRPMSESPH MFDSPPSPQD SLSDQEGYLS S..SSSSHSG
BRF2    APPSPPFSFQ LPRRLSDSP. VFDAPPSPPD SLSDRDSYLS GSLSSGSLSG
TTP     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

        501                    523

BRF1    SDSPTLDNSR RLPIFSRLSI SDD
BRF2    SESPSLDPGR RLPIFSRLSI SDD
TTP     ~~~~~~~~~~ ~~~~~~~~~~ ~~~
```

Figure 19: Alignment of the protein sequence of the three human Tis11 family members BRF1, BRF2 and TTP (SwissProt accession number Q07352, P47974 and P26651, respectively) . Putative phosphorylation sites are shown in red.

family members. Based on this homology, S203 and the other putative MK2 site S283 were further analyzed.

Mutants were constructed, where S203 or S283 were replaced by alanine, yielding BRF1S203A and BRF1S283A (see Materials and Methods). These constructs were used to check, whether mutation of these sites has an effect on the migration of BRF1. The mutants were transfected into HIRc-B cells and stimulated after 24h with insulin. The Xpress-tagged protein was detected by Western blotting using the respective antibody (Invitrogen®). As seen before, insulin did induce an upshift of BRF1 protein (Figure 20, compare lanes 1 and 3, upper panel). This shift was reduced by dephosphorylation of the protein by λ-PPase (lanes 2 and 4) and, therefore, due to phosphorylation. As a control for the insulin stimulation and the dephosphorylation reaction, phospho-ERK1/2 was visualized on the same blot (lower panel). Interestingly, no upshifted, phosphorylated form of the BRF1S203A mutant could be detected, neither in untreated nor in insulin stimulated cells (Figure 20, lanes 5-8). Mutation of S283 (lanes 9-12), on the other hand, showed no difference compared to wild-type BRF1 in respect to migration. This set of data shows that S203 is phosphorylated *in vivo*.

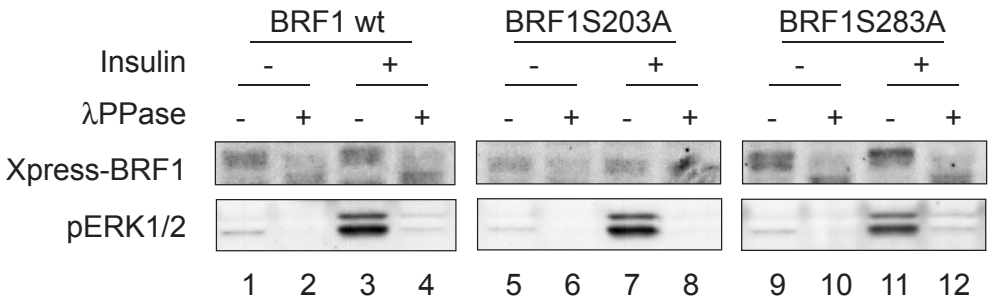


Figure 20: bsd-HisBRF1wt, bsd-HisBRF1S203A and bsd-HisBRF1S283A were transfected into HIRc-B cells. Where indicated, cells were treated with insulin for 15min before harvesting. After λ-PPase-treatment samples were processed for Western blotting and the Xpress-tagged BRF1 (Xpress-BRF1) and activate ERK1/2 (pERK1/2) were visualized by immunoblotting.

For the last set of experiments presented in this section, I would like to acknowledge Min Lu for technical assistance. S203 seems to be an *in vivo* site for BRF1 phosphorylation. To further examine the functional significance of this site, recombinant mutant BRF1 was tested in an *in vitro* decay assay as described in (Schmidlin *et al*., 2004). Addition of BRF1 mutated at S203 (BRF1S203A) alone or at S90, 92 and 203 (BRF1S90/92/203A) to slowC extracts was able to induce degradation of ARE-mRNA similar to BRF1wt. Mutation of S203 did not disturb the decay promoting activity of BRF1 in this assay.

To test, whether phosphorylation at S203 inhibits BRF1 decay promoting activity similar to S92, BRF1S203A and the dox-regulable β-globin-IL3 reporter (used in Figure 13A) were cotransfected alone or in combination with activated kinases m/pPKB and/or MK2EE into NIH3T3 cells, stably expressing the tTA transactivator (B2A2, (Xu *et al*., 1998)/ see Figure 12 for a summary of the Tet-Off system). Transcription of the reporter was turned of by addition of dox and its stability was investigated by northern analysis. In contrast to the *in vitro* data (Schmidlin *et al*., 2004), *in vivo*, BRF1wt partly overcomes the stabilizing effect of PKB. If PKB is combined with MK2, however, BRF1wt is

completely inactivated and the ARE-reporter is stable (Sabrina Leuenberger, PhD thesis, 2004). If phosphorylation of S203 is involved in this inactivation, the BRF1S90/92/203A triple mutant is expected to withstand this inactivation. Indeed, also in the presence of both, activated PKB and MK2, reporter RNA is still rapidly degraded, if the BRF1S90/92/203A was cotransfected. Keeping in mind that S203 is homologous to a known MK2 site in TTP and BRF1S90/92/203A is refractory to PKB/MK2 induced inactivation in decay assays, MK2 seems to be responsible for S203 phosphorylation. However, BRF1S90/92/203A was also resistant to m/pPKB-mediated inactivation, whereas BRF1S90/92A and BRF1S203 were only partly inhibited. This argues that S203 is also regulated by PKB. Further, BRF1S203A does not upshift on SDS-PAGE as compared to BRF1wt, an upshift, which is inhibited by the MEK inhibitor UO126.
To clarify, whether phosphorylation of S203 is mediated by PKB, MK2 or ERK1/2, *in vitro* kinase assays were performed, where rBRF1wt and rBRF1S203A were incubated with activated PKB (Schmidlin *et al.*, 2004), ERK1 (Upstate, #14-439) or with activated p38 alone or together with MK2 (kindly provided by Hermann Gram, Novartis). All tested kinases did phosphorylate rBRFwt. To our surprise, BRF1S203A phosphorylation was comparable to BRF1wt phosphorylation under all tested conditions. One possibility is that in this assay BRF1 becomes phosphorylated at (artificial or physiological) sites different from S203, covering a possible difference between BRF1wt and BRF1S203A. This matter will have to be analyzed in more detail.
Taken together, during these studies, S92 could be identified as major PKB phosphorylation site *in vitro* and *in vivo*. In addition strong evidence for S203 as a phosphorylation site could be accumulated. Both sites seem to be involved in inactivation of BRF1. However, the kinase(s) responsible for S203 phosphorylation and the additional kinase phosphorylating S92 could not be identified yet.

Subcellular Localization of AU-Binding Proteins

Control of protein localization is one way to regulate protein function. For all four AUBPs investigated in this part (HuR, $AUF1_{p37}$, TTP and BRF1), nucleo-cytoplasmic shuttling has been reported before and during these studies (Fan and Steitz, 1998; Murata *et al*., 2002; Phillips *et al.*, 2002; Sarkar *et al.*, 2003). The predominant nuclear localization of HuR, the best-studied example, changes during cell cycle progression (Wang *et al.*, 2000a). During late G1, early S and during G2 phase the cytoplasmic portion of HuR increases. HuR export parallels stabilization of cyclin A and B1 mRNA. In response to UV-irradiation, cytoplasmic HuR levels are also elevated, favoring stabilization of cyclin-dependent kinase inhibitor p21 (Wang *et al*., 2000b) and the small G-protein RhoB mRNA (Westmark *et al.*, 2004). The Tis11 protein family members contain a leucine-rich nuclear export signal that interacts with the nuclear export receptor CRM1. These proteins are nucleo-cytoplasmic shuttling proteins and rely on CRM1 for their export from the nucleus. (Murata *et al.*, 2002; Phillips *et al.*, 2002). Diverse shuttling has been reported for the 4 different AUF1 isoforms. Nuclear export seems to be facilitated by sequences in exon 7, only found in the C-terminal domain of the two larger AUF1 isoforms (Sarkar *et al*., 2003). These findings suggest possible roles for nuclear import and export in the regulation of AUBP activity. Therefore, in the second part of this work, the subcellular localization of the four AUBPs mentioned above was investigated and the question was asked, whether ARE-mRNA stabilization correlates with alterations in AUBP localization.

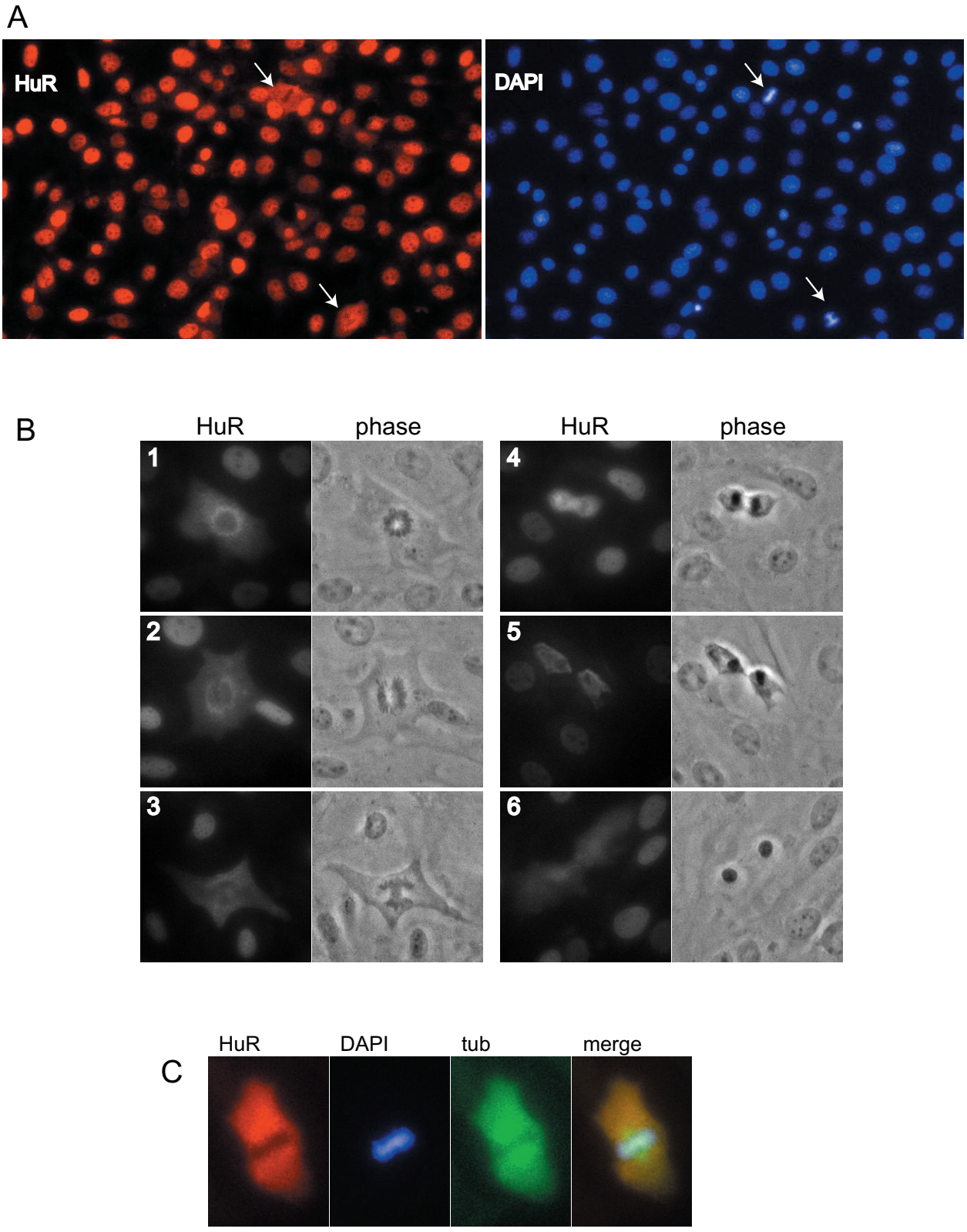


Figure 21: Localization of HuR in NIH3T3 cells. **A:** HuR (immunofluorescence using mouse monoclonal anti-HuR is shown in red) is predominantly localized in the nuclei (DAPI stain in blue), except in mitotic cells (arrows) **B:** Upon entry in mitosis HuR is rapidly exported into the cytoplasm. Cells at different stages of mitosis were stained for HuR and the corresponding phase contrast picture is shown. **C:** Triple staining of a representative cell in metaphase showing HuR (red), DNA (blue), tubulin (green) and the overlay (merge)).

HuR

Endogenous HuR. First, localization of HuR in NIH3T3 mouse fibroblasts was analyzed by immunofluorescence using a monoclonal HuR antibody (3A2). A strong nuclear staining could be observed (Figure 21A). In 1-2% of the cells, HuR was present in the cytoplasm (Figure 21A, see arrows). Further analysis of the cells with cytoplasmic HuR staining revealed that they were entering mitosis, as seen by the beginning condensation of the chromosomes stained with 4',6-diamidino-2-phenylindole (DAPI). Figure 21B shows NIH3T3 cells at different stages of mitosis stained for HuR (panel 1: prophase, 2: late metaphase, anaphase, 3: anaphase 4, 5, 6: telophase). In the phase contrast next to the immunofluorescence picture, the condensed chromosomes can be seen. During the early stage of prophase, HuR is exported from the nucleus and accumulates at the border of the nuclear structure (Figure 21B, panel 1) that is still maintained at this stage of mitosis (see (Aitchison and Rout, 2002) for review on nuclear envelope during mitosis). Accumulation of HuR in the cytoplasm is, therefore, not just the result of the dissolving nuclear membrane.
The localization of HuR during prophase and metaphase resembles the mitotic spindle. Therefore co-localization of HuR with tubulin, a component of this structure (Karsenti and Vernos, 2001), was performed.As seen in Figure 21C, no co-localization could no observed. Whether the export of HuR is of any physiological significance with respect to stabilization of mitosis-regulating transcripts, or whether it only assures even redistribution of the protein after cytokinesis was not further investigated. The fact that the export takes place at the onset of mitosis, when the nuclear structure is still maintained, suggests that HuR is actively exported from the nucleus and that this distribution is of some significance during mitosis. However, experiments with control markers for mitosis were not performed, as the time window of this HuR export is very narrow and it would be difficult to obtain further insight into this phenomenon. Cell cycle dependent changes of HuR localization have been reported by Wang *et al.* (Wang *et al*., 2000a), where stabilization of cyclins A and B1 was paralleled by cytoplasmic accumulation of HuR. This cytoplasmic accumulation of HuR peaked during S phase and HuR was found to reenter the nucleus in G2. In addition, the proportion of cytoplasmic HuR was never higher than 25% of total protein as measured by Western blot. The two phenomena are therefore not directly linked.

myc-HuR: As lined out before, several signaling pathways are known to be involved in stabilizing mRNA. Further, localization of AUBPs seems to be important for their function. Therefore, the hypothesis was tested, whether signaling pathways directly influence the localization of AUBPs and, thereby, regulate their function. To study this for HuR, myc-tagged HuR was cotransfected together with activated kinases of interest into NIH3T3 cells and the subcellular localization was analyzed by immunofluorescence against the myc-tag. Similar to the endogenous HuR, myc-HuR localized to the nucleus (Figure 22A). If activated MEK6 (MEK6DD) was cotransfected together with HuR (Figure 22B), an increase in cytoplasmic HuR levels could be detected. Upon inhibition of MEK6 downstream signaling by the presence of dominant negative p38 MAPK (p38-AGF), HuR stayed in the nucleus (Figure 22C). Export is, therefore, mediated via p38. In this case, a correlation between stabilizing conditions (Ming *et al.*, 2001) and cytoplasmic accumulation of HuR could be observed, strengthening the hypothesis that HuR export from the nucleus plays a role in protecting ARE-mRNA from degradation.

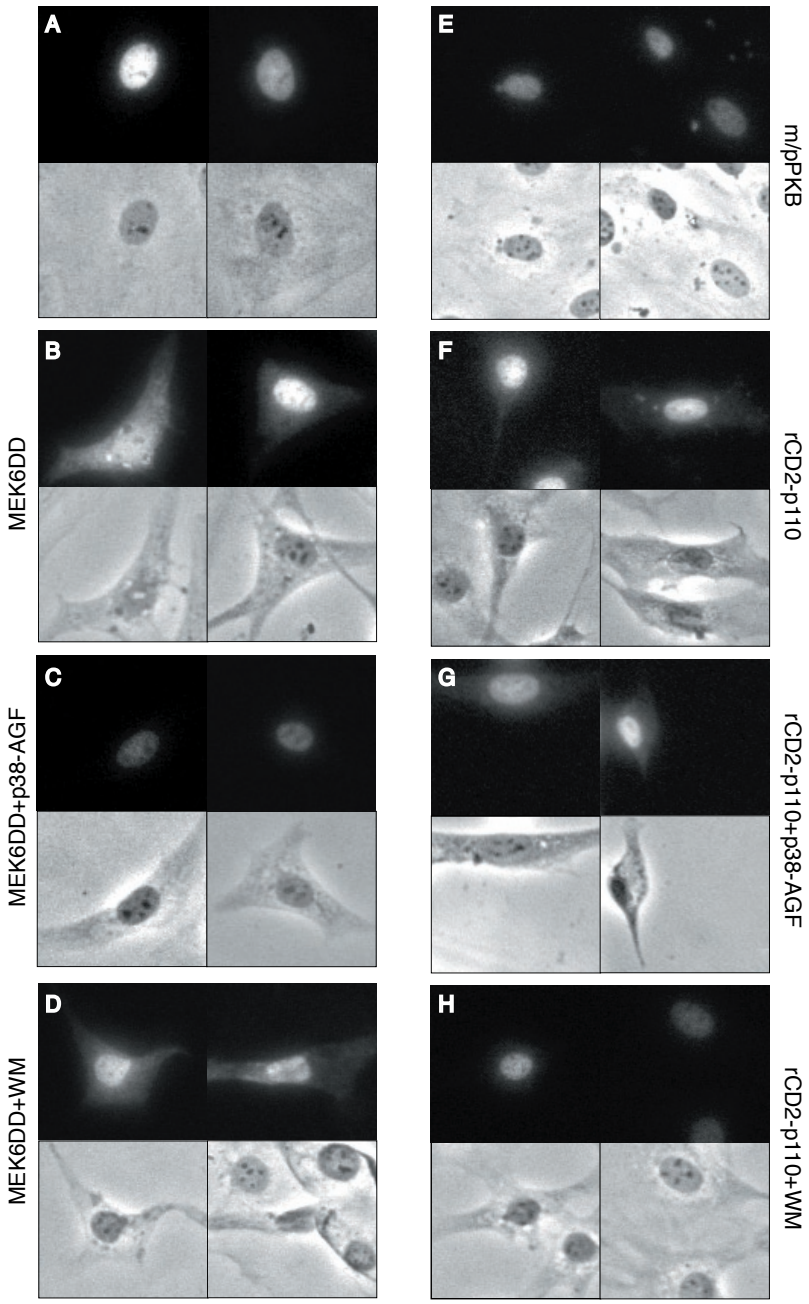


Figure 22: Cotransfection of myc-HuR (pcDNA3.1-HuR-HIS/myc) together with activated or dominant negative kinases as indicated. Localization of myc-HuR was analyzed by immunofluorescence directed against the myc-tag. 2 different cells are shown for each condition with the respective phase contrast picture.

Our lab has also reported that the PI3-K pathway stabilizes ARE transcripts (Ming *et al*., 2001). A constitutively active form of PI3-K, where the cytoplasmic domain of the rat CD2 (rCD2) cell-surface antigen was replaced by the catalytic p110 domain of PI3-K (rCD2-p110) (Reif *et al*., 1996) was transfected together with HuR into NIH3T3 cells. Similar to MEK6DD, activation of the PI3-K signaling pathway increased the levels of cytoplasmic HuR (Figure 22F). This export was not affected by the presence of dominant negative p38-AGF (Figure 22G), arguing that the mechanism leading to MEK6DD induced cytoplasmic accumulation is different from the one induced by rCD2-p110. Inhibition of PI3-K by WM was able to block the PI3-K mediated export of HuR (Figure 22H), but had no effect on MEK6DD (Figure 22D). PKB, a downstream kinase of PI3-K, was also tested. Its stabilizing effect has been discussed in the previous chapter (Schmidlin *et al*., 2004). Cotransfection of HuR together with m/pPKB did not alter the predominant nuclear localization of HuR (Figure 22E). Thus, the PI3-K induced change of HuR localization is not mediated by PKB. As seen in (Schmidlin *et al*., 2004), PKB does phosphorylate and, thereby, inactivate BRF1, leading to stabilization of ARE-mRNA. The PKB upstream effector PI3-K, in addition, seems to regulate mRNA stability by changing the localization of HuR.

12-O-tetradecanoyl-13-phorbol acetate (TPA)-treatment, activating protein kinase C (PKC) is another stimulus, which is known to stabilize ARE-mRNA (Hahn and Moroni, 1994). Cells treated with TPA do also accumulate HuR in the cytoplasm (data not shown). Another drug tested was ActinomycinD (ActD), as it is routinely used to inhibit transcription in mRNA turnover assays. Also treatment of cells with ActD was found to rapidly export HuR from the nucleus (data not shown). This effect has been published by Atasoy *et al.* (Atasoy *et al*., 1998).

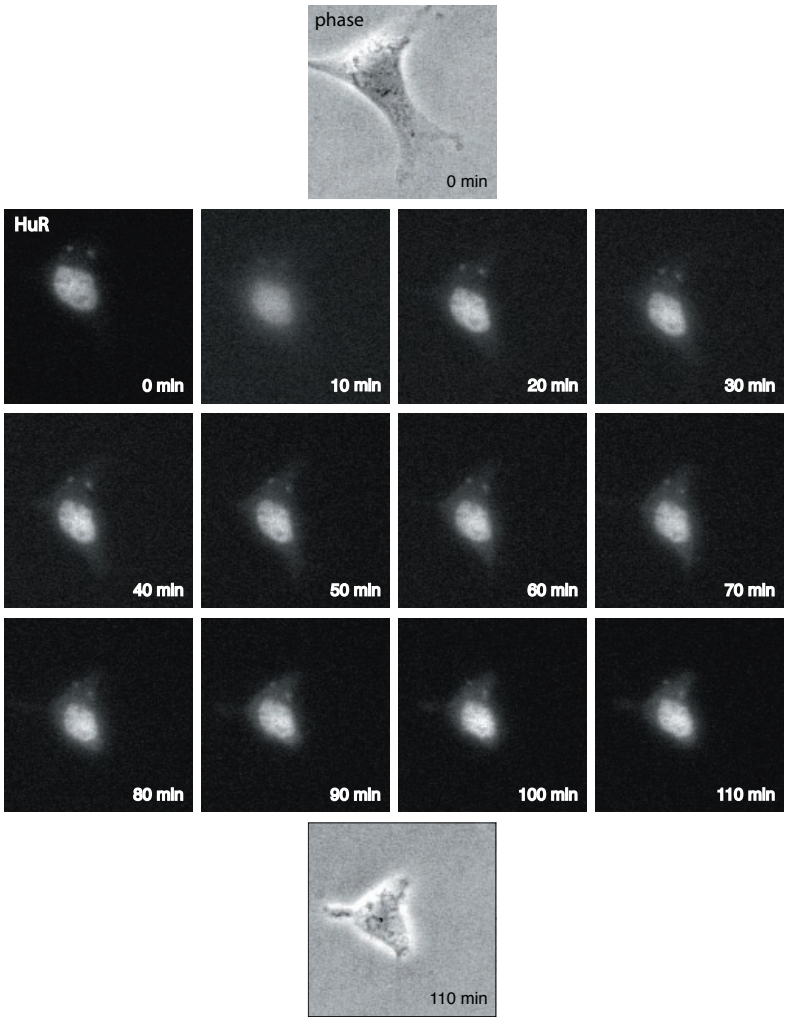


Figure 23: ActinomycinD time course. Localization of GFP-HuR in a living NIH3T3 cell was followed in 10min time intervals after administration of ActinomycinD (5µg/ml).

GFP-HuR: To track HuR in a living cells, green fluorescent protein - HuR fusion protein was constructed (GFP-HuR; see Materials and Methods, Figure 33A) and expressed in NIH3T3 cells. The GFP-HuR protein behaved similar to the myc-tagged HuR under all tested conditions. However, the differences observed were smaller. Although the nuclear localization signal of HuR was able to accumulate the otherwise cytoplasmic GFP in the nucleus, shuttling might be hindered to some extent by the large GFP protein. It was hard to detect differences in localization upon cotransfection of kinases (data not shown). Nevertheless, HuR localization could be visualized in living cells, and again a predominant nuclear localization could also be seen. GFP-HuR was followed in single

cells in 10 minutes time intervals after ActD-treatment. An enrichment of cytoplasmic HuR could be observed over time (Figure 23). HuR was chased out of the nucleus already after 30 minutes. If GFP-HuR relocalization to the cytoplasm is indeed affecting mRNA stability, the results from ActD-chase experiments to measure mRNA half-lives have to be interpreted carefully.
In the following table the immunofluorescence data is summarized in a qualitative way: From all the experiments performed, the results were classified into strong export (++), intermediate export (+) and no export (-). Strong export was attributed, if 25-50% of the protein was exported into the cytoplasm, as estimated by immunofluorescence.

		myc-HuR		GFP-HuR	
Kinase	Drug	Localization	n	Localization	n
		-	10	-	6
	ActD	++	7	++	5
	TPA	++	3	+	1
	Wort	-	3		n.d.
	Rap	-	1		n.d.
	ActD+Wort	+	2		n.d.
rCD2-p110		++	5	+	1
rCD2-p110	Wortmannin	-	2		n.d.
rCD2-p110	Rapamycin	++	1		n.d.
rCD2-p110/p38-AGF		+	2		n.d.
MEK6DD		++	9	+	2
MEK6DD	Wortmannin	++	2		n.d.
MEK6DD	Rapamycin	++	1		n.d.
MEK6DD/p38-AGF		-	3	-	1
MEK6DD/p38wt		++	1		n.d.
p38-AGF		-	3		n.d.
p38-AGF	ActD	++	1		n.d.
PKB		-	2		n.d.

-:no export; +: intermediate export; ++:strong export

Table 4: Summary of immunofluorescence data of HuR

AUF1

myc-AUF1: As our AUF1 antibody did not perform well in immunofluorescence studies, localization of exogenous $AUF1_{p37}$ was followed in a similar set of experiments as described for HuR. myc-tagged $AUF1_{p37}$ was transfected alone or in combination with activated kinases into

NIH3T3 cells and the subcellular localization of the protein was analyzed with immunofluorescence against the myc-tag. AUF1 behaved similar to HuR under all tested conditions: In unstimulated cells $AUF1_{p37}$ was localized mainly in the nucleus. Activation of the p38 MAPK pathway (MEK6DD) as well as the PI3-K (rCD2-p110) pathway induced export of the protein. Also TPA- or ActD-treatment led to export of $AUF1_{p37}$. m/pPKB did not change the nuclear localization of $AUF1_{p37}$.The data is summarized in table 5 again in a qualitative way. In NIH3T3 cells, the same stimuli inducing export of HuR did also lead to export of $AUF1_{p37}$ from the nucleus. Overall, the data obtained with $AUF1_{p37}$ were less conclusive than the results with HuR, as the detected differences were smaller. The parallel export of $AUF1_{p37}$ and HuR may point towards a collaboration of these two RNA binding factors in stabilizing specific sets of mRNA. However, it was not investigated, whether the two AUBPs are also co-regulated in other cells, where AUF1 has been shown to function as a RNA destabilizing protein. Further, it would be interesting to investigate, whether localization of the four different isoforms is regulated differently.

		myc-AUF1		GFP-AUF1	
Kinase	Drug	Localization	n	Localization	n
		-	5	-	6
	ActD	++	3	++	5
	TPA	++	3	+	1
rCD2-p110		++	5	+	1
rCD2-p110	Wortmannin	-	2		n.d.
rCD2-p110	Rapamycin	++	1		n.d.
rCD2-p110/p38-AGF		++	2		n.d.
MEK6DD		+	3	+	2
MEK6DD	Wortmannin	++	2		n.d.
MEK6DD	Rapamycin	++	1		n.d.
MEK6DD/p38-AGF		-	3	+	1
MEK6DD/p38wt		++	1		n.d.
p38-AGF		-	3		n.d.
p38-AGF	ActD	++	1		n.d.
PKB		+	1		n.d.

-:no export; +: intermediate export; ++:strong export

Table 5: Summary of immunofluorescence data of AUF1

GFP-AUF1: To investigate the localization of $AUF1_{p37}$ in living cells, a GFP-$AUF1_{p37}$-fusion protein was constructed (Figure 33B). In transfected cells GFP-$AUF1_{p37}$ localization was similar to the myc-$AUF1_{p37}$ protein, but again the differences observed between the tested conditions were smaller (see table 5). Therefore, the GFP-$AUF1_{p37}$ construct was used to followed AUF1 localization in living cells. GFP-$AUF1_{p37}$ localization was predominantly nuclear. Upon ActD-treatment, $AUF1_{p37}$ was efficiently exported from the nucleus, as seen in a time course of one single cell over 2h (Figure 24).

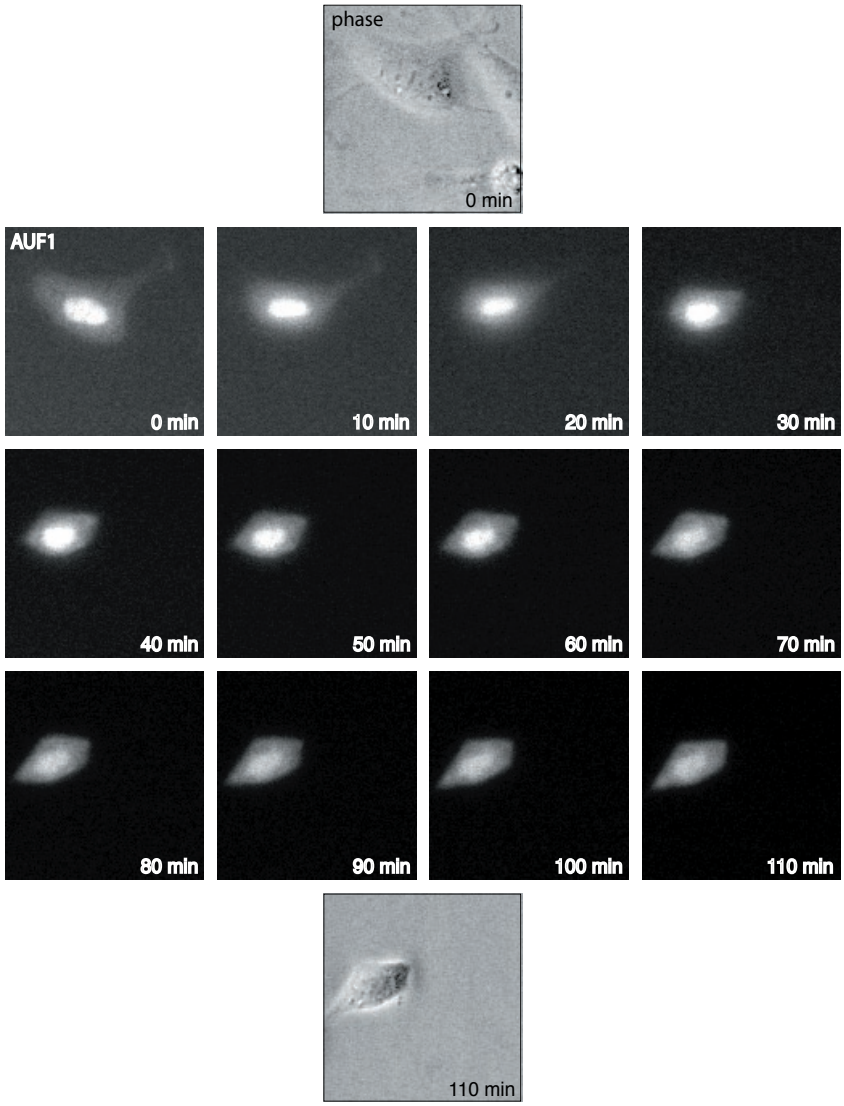
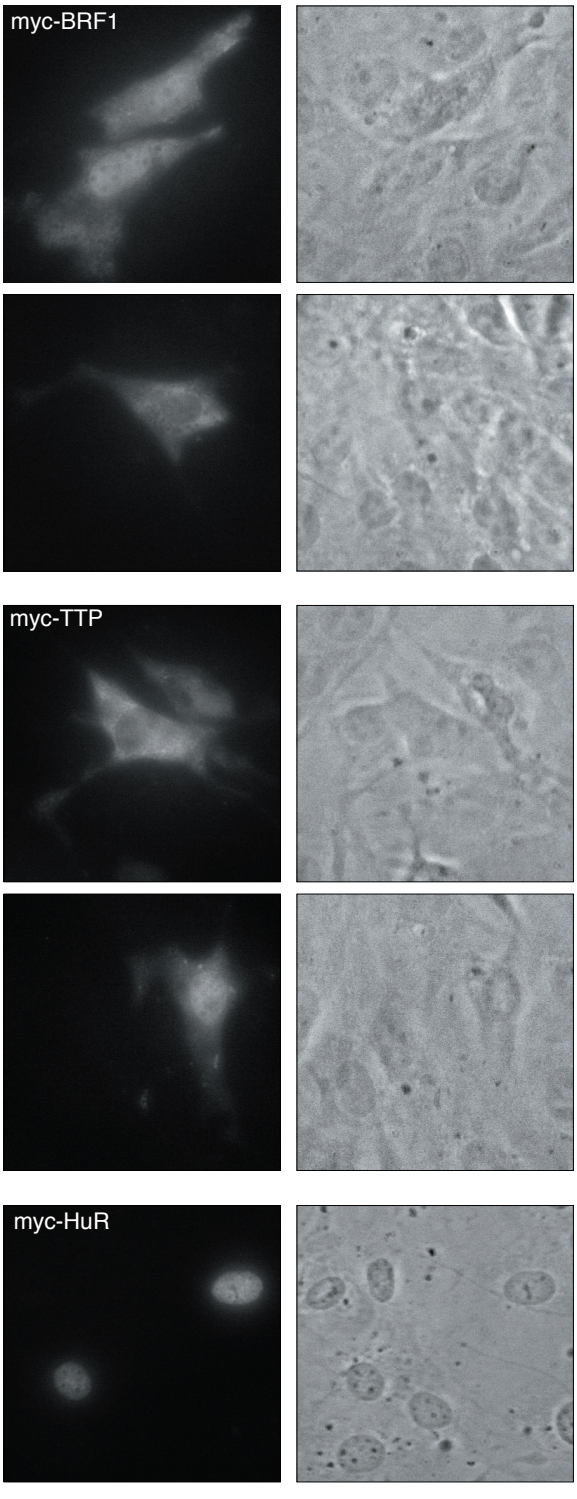


Figure 24: ActinomycinD time course. Localization of GFP-AUF1p37 in NIH3T3 cells was followed in 10min time intervals after administration of ActinomycinD (5µg/ml).

TTP

To obtain high levels of myc-tagged TTP, it was expressed from a tetracycline controllable CMV promoter (pTRE-TTP). With this system constant overexpression of TTP with possible toxic effects (Johnson *et al.*, 2002) could be avoided, as TTP can be induced shortly before analysis. Transfection of pTRE-TTP into B2A2 cells, stably expressing the tTA transactivator (Xu *et al*., 1998), resulted in high expression of myc-TTP, which could be visualized by immunofluorescence. TTP was found to be present in both, the nucleus and the cytoplasm in unstimulated cells (Figure 25 and (Stoecklin *et al*., 2002)). The distribution was about 1:1 with substantial variation. The localization of TTP probably is affected by the cell cycle or the stress states of the cells, which might explain the variations observed.



BRF1

Similar results to TTP were obtained in the case of BRF1. Myc-BRF1 expressed from the same promoter as TTP (pTRE-BRF1) was found in the nucleus and the cytoplasm (Figure 25 and (Stoecklin *et al*., 2002)). Staining for the endogenous BRF1 with a rabbit anti BRF1 antibody (Raineri *et al*., 2004) confirmed the equal distribution (data not shown). However, this staining may to some extent arise from BRF2, which is also recognized by this antibody and is very abundant in unstimulated cells.

The subcellular distribution of TTP and BRF1 did not allow to monitor changes in their localization upon external stimuli or upon activation of signaling cascades. As already a substantial portion of the proteins were present in either cellular compartment and some variability was detected, changes are not likely to significantly influence function. However, it cannot be ruled out that also in the case of TTP and BRF1 changes in the

Figure 25: NIH3T3 cells were transfected with pTRE-BRF1, pTRE-TTP together with the transactivator tTA (pTet-Off) or HuR alone (pcDNA3.1-HuR-HIS/myc) and the subcellular localization of the proteins was visualized by immunofluorescence. Two representative pictures with the respective phase contrast picture to the right are shown.

localization affect function. Due to the small differences detected in the case of the predominantly nuclear proteins HuR and $AUF1_{p37}$ and the variability of TTP and BRF1 localization in unstimulated cells, no further studies were performed to clarify this matter.
Taken together, the experiments above indicate, that the two mRNA stabilizing proteins HuR and $AUF1_{p37}$ are co-regulated in NIH3T3 cells. They are stored in the nucleus and are exported to the cytoplasm, upon activation of the stabilizing p38 and PI3K pathways. In the cytoplasm they stabilize ARE-mRNA. The two destabilizing proteins TTP and BRF1 are present in the cytoplasm, where they can promote constitutive degradation of transcripts. Inhibition of this degradation may either be achieved by phosphorylation and concomitant change of binding partners (see (Schmidlin *et al*., 2004)) or by competition with the stabilizing AUBPs, which accumulate in the cytoplasm in response to mRNA stabilizing stimuli.

Physiological Role of BRF1

BRF1 was functionally cloned in our lab as an ARE-mRNA destabilizing factor. However, very little is known about the physiological role of this protein and its target messages. One report shows an apoptotic effect of BRF1 overexpression (Johnson *et al*., 2000). On the other hand, high levels of BRF1 expression have been found in t(8;21) leukemic cells. Overexpression of BRF1 in myeloid progenitor cells (L-G) induced myeloid cell proliferation and delayed differentiation in response

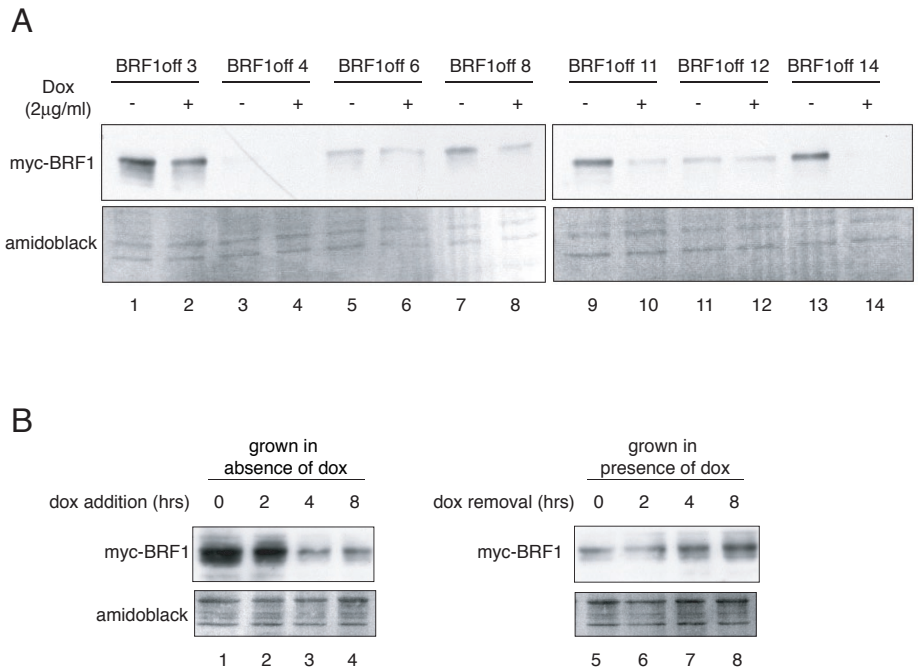


Figure 26: **A:** Testing of BRF1 expression in different clones. Puromycin resistant clones were grown in the presence of dox (+/ 2µg/ml) and dox was removed overnight, where indicated (-). BRF1off11 and 14 (lanes 9, 10 and 13, 14 respectively) were used for further experiments. **B:** Time course of BRF1 repression and induction in BRF1off11. Cells were grown in absence (lane 1-4) or presence (lane5-8) of dox and the drug was then added or removed for indicated time periods.

to granulocyte colony-stimulating factor (G-CSF). These results suggest that the high expression of BRF1 contributes to AML1-MTG8-mediated leukemogenesis (Shimada *et al*., 2000). These data show a dual role of BRF1 in both, apoptosis and cell proliferation. To further understand this controversial set of data, the effect of BRF1 expression was studied in NIH3T3 cells.

Construction of a Doxycycline-Repressible Cell Line

As mentioned above, BRF1 might induce apoptosis and might therefore be toxic, when overexpressed. A system was required, where BRF1 expression could be turned on in a given time window. This was achieved by constructing a doxycycline (dox) / tetracycline (tet) inducible cell line (Gossen and Bujard, 1992), which would allow to study BRF1 effects in a monoclonal situation and should circumvent any possible toxic effect of BRF1 overexpression. The Tet-Off system has already been introduced in Figure 12.

B2A2 cells stably express the tTA transactivator (Xu *et al*., 1998). Into a subclone from this cell line (clone 23) BRF1 was transfected under the control of the tet-responsive promoter described above (pTRE-BRF1 see Materials and Methods) together with a plasmid carrying a puromycin resistance gene (pMX-puro) in a 10 to 1 ratio. The transfected cells were selected with puromycin (2µg/ml). Surviving cells were tested by Western blot analysis for dox repressible BRF1 expression. The clones were grown in the presence of dox. 16h after dox removal, the cells were harvested and BRF1 expression was analyzed. 2 clones (named BRF1off11/BRF1off14) with low background and strong induction of BRF1 expression were used for further analysis (see Figure 26A, lanes 9, 10 and 13, 14). If the cells were grown in absence of dox, the high BRF1 expression could be turned down within 4h after addition of dox (Figure 26B, lanes 1-4). Cells grown in the presence of dox expressed background levels of BRF1, which could be further induced by removal of dox (Figure 26B, lanes 5-8). The longer exposure of the Western blot revealed some leaky expression of BRF1 also in the presence of dox.

Role of BRF1 in Cell Cycle Control

Many cyclin and growth factor mRNAs contain AREs in their 3'UTR, which makes them putative targets for BRF1 mediated degradation. Therefore, it seemed plausible to ask, whether BRF1 levels play a role in cell cycle control. The cell cycle distribution of cells was analyzed by staining cells with propidium iodide (PI), a DNA intercalating dye, giving a fluorescent signal proportional to the DNA content. By fluorescence assisted cell scan (FACS), the signal intensity was measured. To analyze changes in the cell cycle distribution, cells needed to be synchronized. This was done by serum-starvation. 24h after FCS-removal, approximately 90% of the cells accumulated in G0/G1. In Figure 27, panel 1 a normal distribution of untreated B2A2 cells can be seen. After starvation, only one population of NIH3T3 cells corresponding to G0/G1 cells could be detected (panel 2). Upon addition of 10% serum, the cells started to progress through the cell cycle synchronously, entering S phase after approximately 16h (Figure 27 panel 3, reduced height of the G0/G1 peak) and reaching G2 after about 20-24h (panels 4 and 5). The presence of dox did not affect the synchronization, as the green line (no dox) overlaps with the blue curve (plus dox).

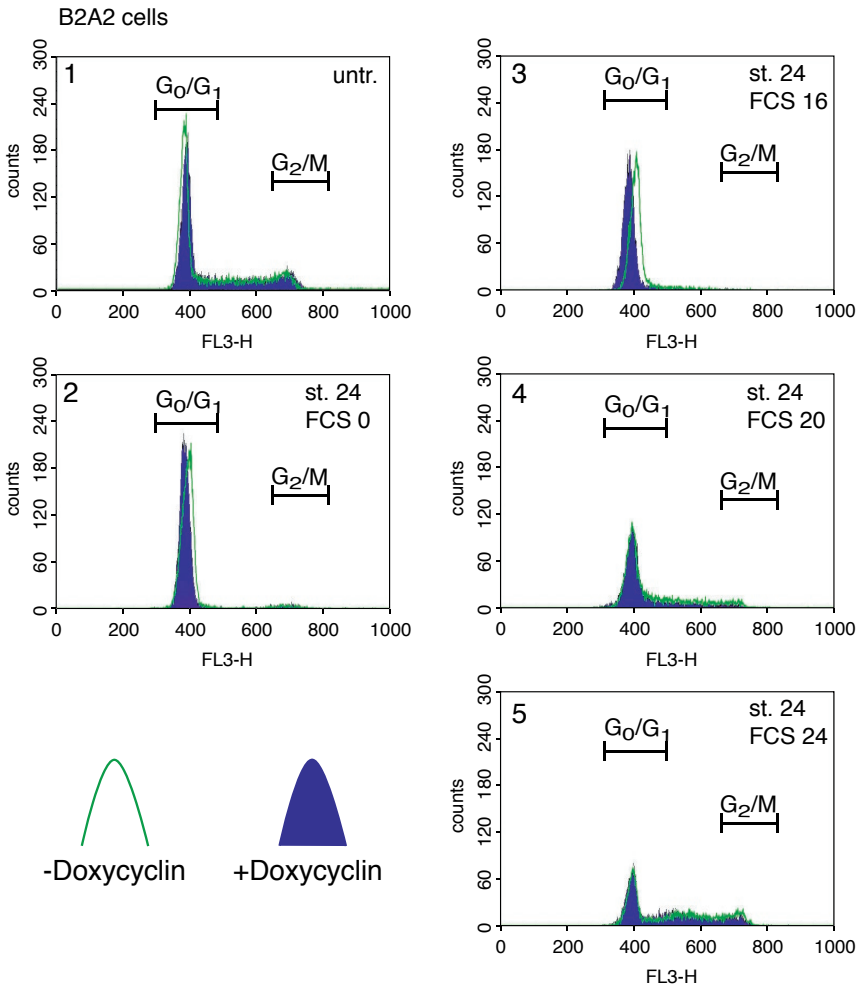


Figure 27: FACS profiles of B2A2 cells stained with PI in absence (green line) or presence of dox (blue curve). G1: Cells in G1 phase; G2/M cells in G2/M phase; Untr.: untreated cells; st. 24: starved for 24 h; FCS: addition of FCS for indicated hours.

With this synchronization protocol established in NIH3T3 cells, cell cycle progression of the BRF1 overexpressing cells was now analyzed in the presence or absence of dox, resulting in lower or higher levels of BRF1 respectively. The BRF1off11 and BRF1off14 cells were passaged in presence of dox and dox was removed, where mentioned, when the cells were splitted for the experiment 24h before starvation. BRF1off11 or BRF1off14 cells were starved for 24h and the G0 arrest was subsequently released by the addition of 10%FCS in presence (repression of BRF1 expression) or absence of dox (BRF1 overexpression). After starvation (Figure 28, panel 2), a high percentage of the cells were in G0/G1. In the absence of dox (green line) the cells entered the S phase more rapidly than the control cells (Figure 28, panels 3-6: the shoulder to the right of the G0/G1 peak, representing the S phase cells, appears faster, and the G0/G1 population is reduced more quickly in absence of dox). This effect was consistently observed in both cell lines tested and was absent in the control B2A2 cell line (Figure 27 panels 3-5), arguing that overexpression of BRF1 is driving G0/G1 to S transition. However, the difference, although reproducible, was rather small, with a maximum of about 20% difference of G0/G1-cells 16 hours after starvation release.

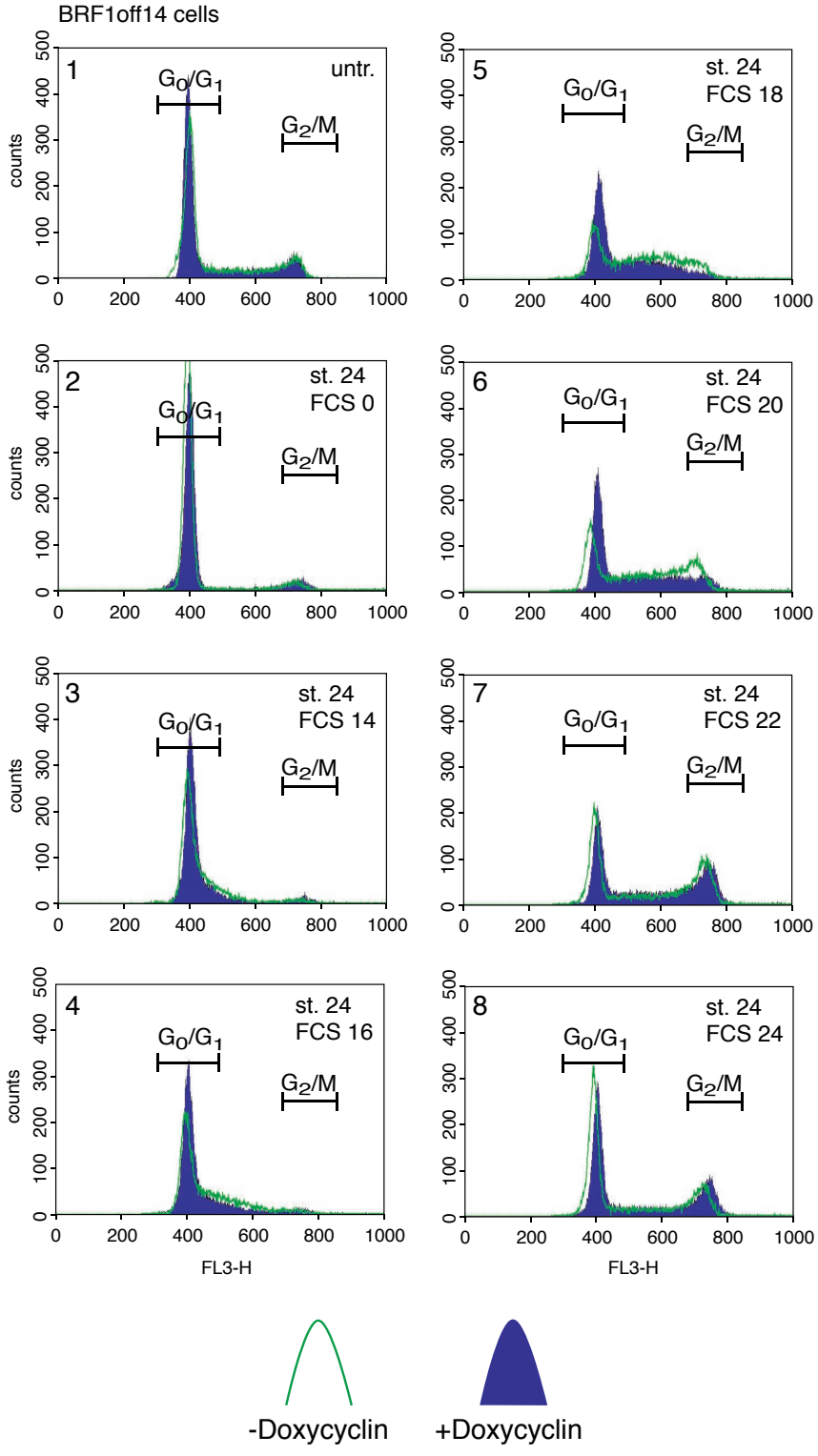
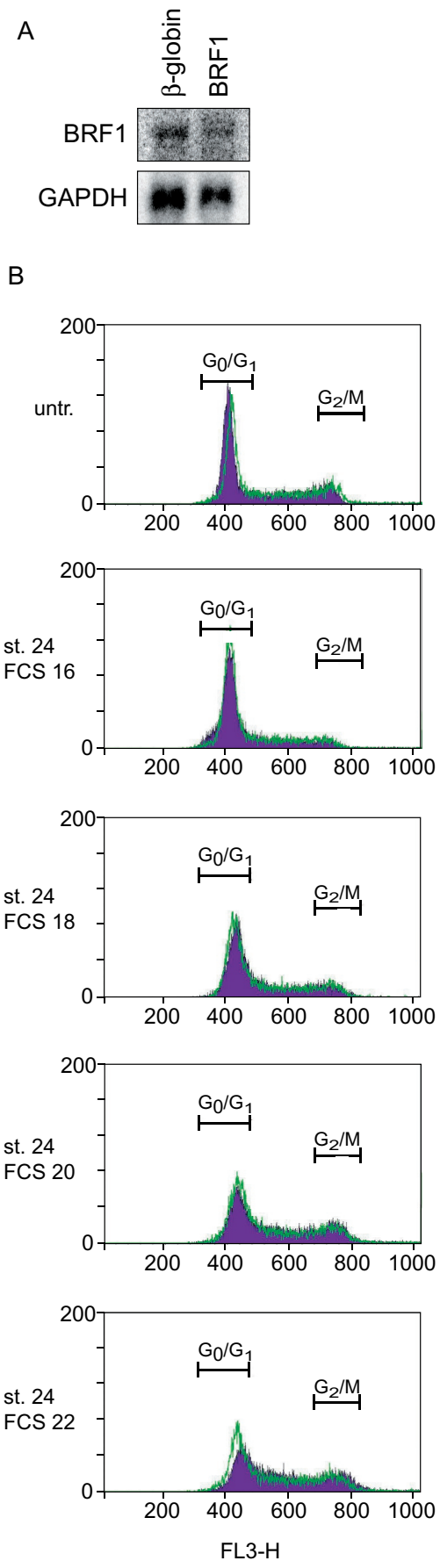
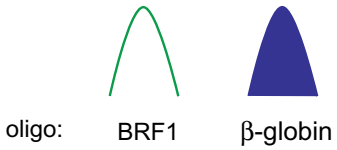


Figure 28: FACS profiles of BRF1off14 cells stained with PI in absence (green line) or presence of dox (blue curve). G1: Cells in G1 phase; G2/M cells in G2/M phase; Untr.: untreated cells; st. 24: starved for 24 h; FCS: addition of FCS for indicated hours.



To further confirm this observation, BRF1 was downregulated by RNAi. siRNA directed against nucleotides 130-148 of BRF1 or nucleotides 245-263 of the unrelated β-globin were transfected by lipofection into NIH3T3 cells. 24h after transfection, FCS was removed for another 24h to synchronize the cells. At this time point, RNA was extracted to measure the amount of BRF1 message by northern blotting (Figure 29A). Compared to the β-globin transfected extracts, siRNA against BRF1 was able to downregulate mRNA levels of BRF1 (25% reduction, if standardized to the GAPDH loading control). After release of the cells from starvation, the cell cycle progression was analyzed as before. Downregulation of BRF1 slowed down the transition of 3T3 cells from G0/G1 to S phase compared to the negative control β-globin, seen best 22h after starvation (Figure 29B, siRNA against BRF1 is shown with the green line). However, the effect observed was minimal. This could be explained by the fact, that downregulation of BRF1 was not very efficient and transfection followed by starvation led to a substantial number of dying cells, with mostly untransfected cells, which survived. This is also the reason, why not enough cells could be harvested for FACS analysis before readdition of FCS.

Figure 29: **A:** BRF1 and GAPDH mRNA levels were measured by northern blot analysis 48h after transfection of siRNA against BRF1 or β-globin. **B:** B2A2 cells transfected with siRNA against β-globin or BRF1. After 24h the cells were starved for another 24h before 10% FCS was added for indicated time periods. FACS profiles of the PI stained cells are shown. G0/G11: Cells in G0/G1 phase; G2/M cells in G2/M phase; Untr.: untreated cells; st. 24: starved for 24 h; FCS: addition of FCS for indicated hours.



To confirm the above result, the effect of BRF1 overexpression in BRF1off11 or BRF1off14 cells on [^{3}H]-thymidine incorporation was investigated. This method allows to quantify the amount of ongoing DNA synthesis. In this assay no statistically significant difference could be detected, dependent on BRF1 levels (data not shown).
Taken together, the experiments indicate that BRF1 at high levels accelerates the transition of NIH3T3 cells from G0/G1 to S phase, whereas reduced levels seem to have the opposite effect. The differences observed were reproducible but minimal, making it difficult to further investigate this phenomenon. It cannot be stated, whether this effect is due to destabilization of a specific set of ARE-mRNAs, which inhibit cell cycle progression, or whether the effect seen is indirect. Due to the mentioned difficulties this project was discontinued at this stage.
Overall, weak evidence is presented in this thesis that BRF1 is involved in cell cycle progression, but no clear conclusions on the physiological role of BRF1 can be drawn, based on these data. The work on regulation of BRF1 activity was more successful. Two regulatory sites could be identified in BRF1 (S92 and S203). Together with the localization data of HuR, these findings provide us with a better understanding of the regulation of ARE-mRNA turnover.

Discussion

Regulation of BRF1 Activity by Different Signaling Pathways

In this work, different aspects of AU-binding protein regulation and function were analyzed. These proteins are involved in the turnover of ARE-containing mRNA. Today only few AUBPs are known (see table 3, introduction), but analysis of the human genome revealed that approximately 8% of all mRNAs contain an AU-rich element in their 3'UTR (Bakheet *et al.*, 2001). How does a cell control the stability of so many transcripts, encoding functionally completely diverse proteins, with only a handful of binding proteins? In addition, some of these AUBPs seem to have only a restricted set of target mRNAs, as shown by the TTP knockout mouse, in which only stabilization of GM-CSF and TNFα message was detected (Taylor *et al.*, 1996; Carballo *et al.*, 2000). One solution to this problem might be that, in addition to the ARE, secondary elements play a role. This hypothesis is strengthened by the fact that it is not possible to deduce the activity of an AU-rich element based on its primary sequence. Further, a growing number of AUBP-interacting proteins and signaling pathways modifying the AUBP function shed light on the complex regulatory network, which is needed to precisely control activity and specificity of the AUBPs. The goal of this work was to better understand this regulation of AUBP activity with focus on BRF1.

PKB phosphorylates BRF1 at S92. BRF1 has been functionally cloned in our lab (Stoecklin *et al.*, 2002) and has been shown to promote ARE-dependent mRNA degradation *in vitro* and *in vivo*. According to its protein sequence, BRF1 contains multiple putative phosphorylation sites, where its activity might be regulated (Figure 8). Among others, BRF1 contains two overlapping PKB consensus sites at S90 and S92. By mass spectrometry, S92 was confirmed to be phosphorylated by PKB (Schmidlin *et al.*, 2004). However, these data were obtained, using recombinant BRF1 phosphorylated *in vitro*. To confirm that S92 phosphorylation is physiologically relevant, an antibody was raised against a phospho-peptide spanning the region of S92 (Figure 9/10). With this antibody, phosphorylation of endogenous BRF1 was investigated. Indeed, S92 phosphorylation could also be detected *in vivo* after transfection of activated PKB (m/pPKB). Also PKB activation by a more physiological stimulus, such as insulin, can trigger phosphorylation of BRF1 at S92, indicating that this site is a PKB target *in vivo*. PKB activation of insulin was very poor in NIH3T3 cells. Therefore, BRF1 phosphorylation was also investigated in HIRc-B rat fibroblast cells, overexpressing the human insulin receptor. In these cells, a prominent S92 phosphorylation could be detected upon insulin stimulation. Although BRF1 expression was found to be induced by insulin (Corps and Brown, 1995), the concentration used in the experiments described in this thesis did not alter BRF1 levels under the tested conditions.

A WM-insensitive kinase also phosphorylates BRF1 at S92. In contrast to NIH3T3 cells, where insulin induced PKB activation and phosphorylation of S92 in BRF1 is only weak, but WM-sensitive, in HIRc-B cells S92 phosphorylation could only partly be inhibited by WM (Schmidlin *et al.*, 2004). This suggests that, in addition to PKB, another kinase is likely to phosphorylate the same site. In the HIRc-B cells the response to insulin is much stronger than in NIH3T3 cells, as seen by the activation of PKB (compare Figures 10 and 15). In addition to the PKB pathway, in HIRc-B cells insulin stimulation also strongly activates other pathways, including the ERK1/2 and p38 pathways (Figure 15), leading to phosphorylation of BRF1 in a WM-insensitive manner. A single serine can be phosphorylated by more than one kinase. The nuclear factor CREB, for example, is phosphorylated at S133 by several different kinases, including PKA, PKB and other AGC kinases (Johannessen *et al.*, 2004). In the case of S92 in BRF1, the additional kinase seems to be inhibited by the MEK1/2 inhibitor UO126 (Figure 15), indicating that the downstream kinases ERK1/2 are involved in this phosphorylation event. S92 is not placed in the context of an ERK consensus site (Davis *et al.*, 1993), which would require a proline following the phosphorylated serine. However, also the PKB consensus is not optimal (lacking a hydrophobic residue after the serine), but, nevertheless, phosphorylation by PKB is very efficient. ERK target proteins have been found to share at least one of two common docking motif (Jacobs *et al.*, 1999): the D-domain (K/R-X-X/K/R-K/R-$X_{(1-4)}$-L/I-X-L/I), which can be placed rather remote from the actual phosphorylation site, or the DEF site (F-X-F-P) in the proximity of the phospho-site. Interestingly BRF2 contains a D-domain in its N-terminus (see Figure 19 (amino acids 17-27)), the Lysines being conserved in BRF1. Again, this pattern only serves as a hint and no proof, but it seems as if phosphorylation of S92 by ERK1/2 would be possible. However, further experiments need to be done to identify the respective kinase.

Phosphorylation of S92 inhibits the decay promoting activity of BRF1. *In vitro* decay experiments revealed that phosphorylation of S92 in BRF1 by PKB inhibits its decay promoting activity. Mutating S92 to alanine conferred resistance to this inactivation, showing that S92 is an important regulatory site. In addition, decay experiments in HIRc-B cells showed that, under conditions of BRF1 phosphorylation, such as transfection of activated m/pPKB or insulin stimulation, an ARE-containing reporter transcripts and endogenous VEGF message are stabilized. Further, it could be shown that the insulin induced stabilization is dependent on PKBα, as in PKBα -/- cells, the ARE-mRNA was generally less stable, and could not be stabilized upon insulin stimulation (Schmidlin *et al.*, 2004). These data indicate that PKB mediated stabilization of ARE-mRNA involves inactivation of BRF1 by S92 phosphorylation.

BRF1 interacts with 14-3-3 in a phosphorylation-dependent manner. BRF1 inhibition seems to be accomplished by phosphorylation dependent binding of BRF1 to the scaffold protein 14-3-3. Interaction of 14-3-3 and BRF1 has already been reported by others (Bustin and McKay, 1999; Johnson *et al.*, 2002) and was shown in our lab to depend on S92 phosphorylation: Recombinant BRF1 was able to pull down 14-3-3 from slowC cell extracts, only, if it was pre-incubated with PKB. Further, 14-3-3 could be co-immunoprecipitated together with exogenous BRF1 from COS7 cells if S92 is phosphorylated (Schmidlin *et al.*, 2004). In a very recent experiment, Brigitte Gross in our lab was able to co-immunoprecipitate endogenous 14-3-3 with a BRF1 antibody, for the first time confirming endogenous 14-3-3-BRF1 interaction. How the BRF1-14-3-3 interaction abolishes

the decay promoting activity of BRF1 is not known yet. Phosphorylation of BRF1 does not seem to affect binding of BRF1 to the mRNA (Schmidlin *et al.*, 2004), neither does it alter the localization of BRF1 in the cell (data not shown). Probably, BRF1 coupled to 14-3-3 is no longer able to recruit the exosome to the mRNA. If BRF1 is still bound to mRNA in its inactive state, it might exert a stabilizing effect on the message, by hindering the exosome to access the transcript. If this is true, BRF1 may also be looked at as a stabilizing protein, which is turned into a destabilizing factor upon dephosphorylation. Experiments to clarify these points still need to be done. In the case of $AUF1_{p40}$, phosphorylation changes the conformation of the bound mRNA, thereby, probably, altering the composition of the ribonuleo-protein complex (Wilson *et al.*, 2003a; Wilson *et al.*, 2003b).
It has been reported by others that efficient 14-3-3 binding requires two phospho-sites on a protein. In the case of TTP, phosphorylation of S54 and S178, both being targets of MK2, is required (Chrestensen *et al.*, 2003; Stoecklin *et al.*, 2004). It is very likely that BRF1, too, requires another phosphorylated site in addition to S92 for efficient 14-3-3 binding. This idea is underlined by the fact that, *in vivo*, overexpressed BRF1 can only partly be inactivated by active PKB alone, but needs in addition at least one other kinase for full inactivation (see Figure 30). Eventually, under conditions of double phosphorylation BRF1-14-3-3 interaction is more stable. The existence of this second phospho-site still needs to be confirmed and is the focus of ongoing work in our lab.

BRF1 is phosphorylated at sites different from S92. BRF1 can be detected as multiple bands on Western blot. These bands represent different forms of BRF1, as they can be seen with exogenous BRF1, visualized with an antibody against a tag, and they are also found with endogenous BRF1. Upon arsenite stimulation of NIH3T3 cells and insulin-treatment of HIRc-B cells all bands are condensed to one slowly migrating form. Phosphatase-treatment reduces the different forms to one fast migrating band. These findings, together with the multitude of putative phosphorylation sites found by bioinformatics and the fact that phosphorylation of BRF1 at S92 does not change the migration of BRF1 on Western blots, strongly suggest that BRF1 is phosphorylated also at sites different from S92. Interestingly, differently phosphorylated forms of BRF1 are present in unstimulated (and starved) cells. The function of these different forms of BRF1 is still a field of speculation. Recently, it was shown in our lab that treatment of PB-3c mast cells with the Protein Phosphatase 2A (PP2A) inhibitor okadaic acid stabilizes ARE-containing reporter transcripts (Benjamin *et al.*, 2004). Under these conditions BRF1 is hyperphosphorylated (Don Benjamin, unpublished data). One hypothesis, therefore, would be that fully phosphorylated BRF1 is inactive and might be activated by dephosphorylation. In unstimulated cells, a fraction of BRF1 protein is stored in an inactivated, phosphorylated state ready for rapid activation. It is also possible that the different phosphorylation forms exert different functions, by, e.g., blocking the access of the decay machinery to certain mRNA. Another possibility would be that differently phosphorylated BRF1 populations target different sets of mRNAs. As shown in Figure 25, BRF1 is localized throughout the cell in unstimulated cells. Under the same conditions multiple phosphorylation forms of BRF1 can be detected. Further, it has been reported that BRF1 localizes to stress granules upon arsenite-treatment (Georg Stoecklin, personal communication). Therefore, localization of BRF1 might be regulated by phosphorylation.
Comparision of the protein sequence of all three Tis11 family members revealed only one conserved, putative phosphorylation site (Figure 19), namely S203 in BRF1. Therefore, S203 was mutated

to alanine (BRF1S203A). Interestingly BRF1S203A shows a different migration pattern from BRF1wt, lacking the hyperphosphorylated, slowly migrating band. Migration of the mutant does not change upon insulin stimulation in HIRc-B cells (Figure 20). These data strongly suggest that S203 is an *in vivo* phosphorylation site of BRF1. Cotransfection decay assays confirm the importance of S203: In contrary to the BRF1wt protein, the BRF1S203A can only partly be inactivated by combination of MK2EE and m/pPKB and the BRF1S90/92/203A triple mutant is resistant to this inactivation. This finding indicates that MK2 is the kinase responsible for S203 phosphorylation. However, the partial resistance of BRF1S90/92A to inactivation by PKB alone can be enhanced by mutating S203 in addition, indicating that PKB affects this site. Hence, the kinase responsible for S203 phosphorylation still remains to be identified. The fact that rasV12 is still able to inactivate BRF1S90/92/203A indicates that there are even more sites, which play a role in inactivation of BRF1.

Together, the data discussed here, suggest a model for BRF1 regulation, which is shown in Figure 30: BRF1 exists in several differently phosphorylated forms. Activation of PKB, e.g. by insulin or another stimulus, leads to phosphorylation of BRF1 at S92. This event can be inhibited by WM. Upon insulin stimulation also ERK1/2 are activated and one of these kinases seems to phosphorylate BRF1 at S92 in a WM-insensitive way. Phosphorylated S92 displays a 14-3-3 binding consensus (R-S-X-pS-X-P) (Tzivion and Avruch, 2002), missing only the P at position +2. Phosphorylation of S92 leads to interaction of BRF1 with 14-3-3. 14-3-3 is known to bind as a dimer to 2 phospho-serine/threonine motifs. Therefore, the interaction is probably strengthened by a second phosphorylation event, as it has been reported for TTP. In addition to S92, also S203 is a phosphorylation site. In TTP, phosphorylation of the homologues site to S203 was shown to be mediated by MK2 and enhance 14-3-3 binding (Stoecklin *et al.*, 2004). Whether the same is true for S203 in BRF1 remains to be investigated. S203 is not placed in the context of a 14-3-3 binding motif, however, also the homologues site in TTP is not, and still, binding could be shown. Further, BRF1 is likely to be phosphorylated also at sites different from S92 and S203. Data obtained from insulin (Figure 14) or arsenite (Figure 16) stimulation experiments indicate that the ERK1/2 and the p38 pathways are involved in these phosphorylations. The functional significance of these phosphorylation events is not clear yet. In the current model multiple signaling pathways converge on BRF1 and regulate its activity.

However, we have to take in account, that BRF1 is only one player in the game. AREs can be bound by multiple AUBPs and the fate of the RNA depends on its affinity to one specific set of AUBPs, their activation status and their local concentration. Despite the homology of the three destabilizing Tis11 family members BRF1, BRF2 and TTP, they seem to be differentially regulated. S203 is the only putative phosphorylation site present in all three proteins (Figure 19). Also, their expression is regulated differentially. Upon leukemia inhibitory factor (LIF) removal in embryonic stem (ES) cells, which triggers differentiation, TTP levels increase, whereas BRF1 and BRF2 levels decrease (Ines Raineri, unpublished data) or upon LPS stimulation in RAW macrophages TTP and BRF1 expression is induced, whereas BRF2 is also expressed in unstimulated cells (Figure 17). Further, ARE-mRNA cannot only be stabilized by inactivation of decay promoting AUBPs, but also by activation of stabilizing components, such as HuR. In addition, regulation of yet unknown factors might modulate BRF1 activity. Increasing evidence indicates that mRNA turnover is tightly coupled

to translation and that *cis*-elements, such as the poly(A)-tail and the cap, play an important role in mRNA decay. To understand the complex interplay of all factors involved in the control of ARE-mediated mRNA turnover, a lot more work needs to be invested.

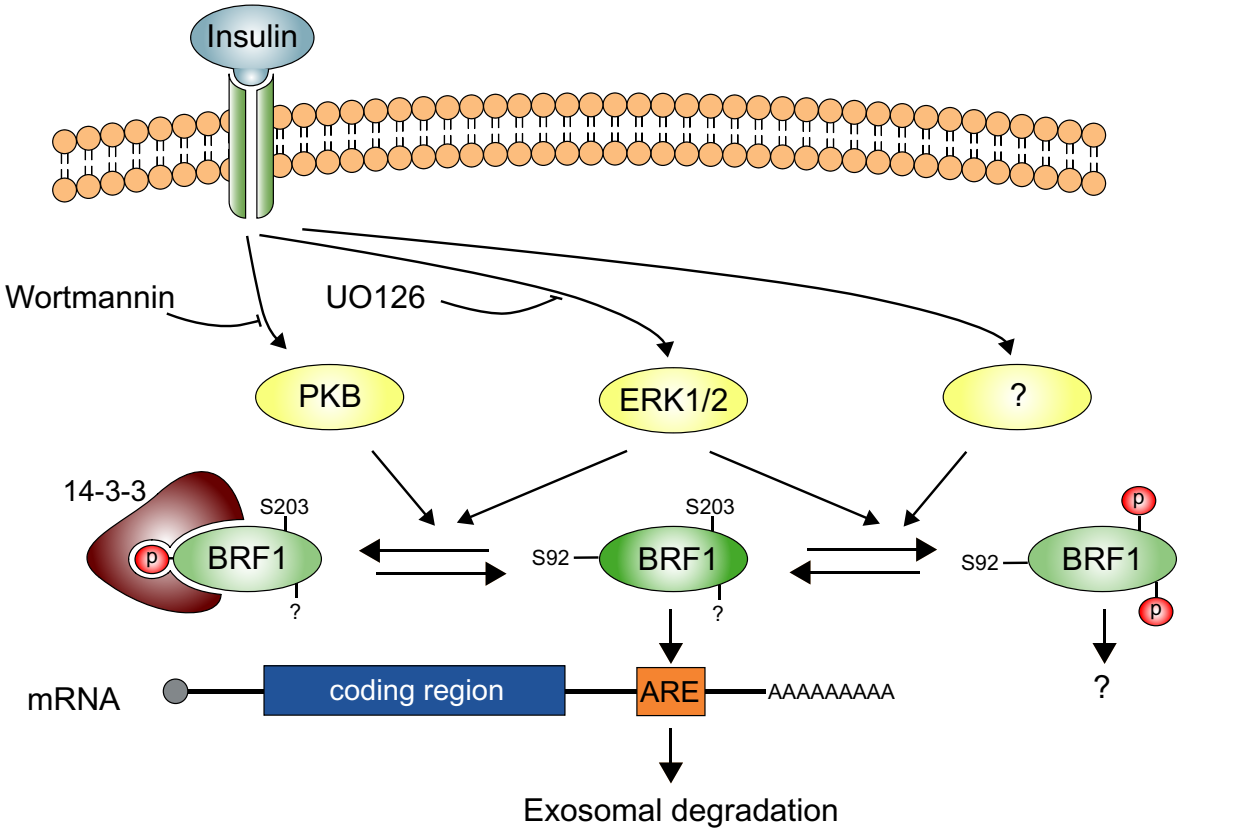


Figure 30: Model of BRF1 regulation. See text for details

Subcellular Localization of AUBPS

The two stabilizing AUBPs HuR and AUF1$_{p37}$ are exported from the nucleus under conditions of ARE-mRNA stabilization. In accordance to previously published data, HuR and AUF1 were found to be shuttling proteins. Their predominant nuclear localization changes rapidly in response to different stimuli. These stimuli include activation of PI3-K, MEK6 but not PKB. Data by Ming *et al.* (Ming *et al.*, 2001) showed that the p38 pathway, but not the PI3-K pathway, in combination with HuR overcome the strong destabilizing activity of TTP. These data suggest that HuR is regulated via the MEK6/p38 axis. The stabilizing activity of PI3-K, on the other hand, seems to be mediated mainly via PKB and inactivation of BRF1 (Schmidlin *et al.*, 2004), which cannot antagonize another destabilizing activity by TTP, explaining the dominant effect of TTP over PI3-K. p38, on the other hand, seems to activate HuR activity, which can push the system in the direction of stabilization. However, the fact that PI3-K also affects HuR localization may be explained by another PI3-K downstream pathway different from PKB, e.g. the PKC pathway. TPA, a strong activator of PKC, stabilizes ARE-mRNA (Hahn and Moroni, 1994) and is also able to translocate HuR to the

cytoplasm, strengthening the hypothesis that PKC is involved in shuttling of HuR and that PI3-K affects HuR localization rather via PKC than via PKB.
Another signal affecting HuR localization has been reported by Wang *et al.* (Wang *et al.*, 2002): AMP-activated kinase inhibits shuttling of HuR to the cytoplasm. In this study, the researchers further claim that the p38 and PI3-K pathways do not affect the subcellular distribution of HuR in RKO cells. These conclusions are based on experiments using inhibitors of p38 and PI3-K. As in NIH3T3 cells HuR predominantly localizes to the nucleus, a further decrease of cytoplasmic levels, which would be expected using the inhibitors, could not be measured. However, it may well be that not all kinases are of equal importance in different cell types. Another interesting observation is that HuR is exported early in mitotic cells. This may provide a new link between ARE-mRNA turnover and mitosis.
$AUF1_{p37}$ localization was affected by the same stimuli as HuR. MEK6, PI3-K, but not PKB did induce translocation of AUF1 to the cytoplasm. The role of AUF1 in mRNA turnover has not been clarified yet. In NIH3T3 cells, transfection of the p37 isoform did result in stabilization of ARE-mRNA (Ming, unpublished data). The co-regulation of AUF1 and HuR localization seems to assure efficient stabilization of target mRNA. Recently, it has been shown that AUF1 and HuR target distinct but also common sites on the same mRNA (Lal *et al.*, 2004). It is well possible that the two proteins cooperatively stabilize one particular set of mRNA. It would be interesting to investigate, whether AUF1 localization is regulated similarly in cells, where an mRNA destabilizing activity has been reported for this protein. In addition, the different isoforms seem to exert distinct effects on mRNA and nuclear export is facilitated by exon 7, which is only present in the two larger isoforms (Sarkar *et al.*, 2003). In this thesis, only the localization of the p37 isoform was investigated. Therefore, it would also be interesting to compare the localization of the 4 isoforms in response to different stimuli.
Treatment of cells with ActD did also lead to HuR and $AUF1_{p37}$ export. The nuclear export of both proteins takes place in less than half an hour, observed in living cells. This is of particular interest, as mRNA turnover is routinely measured by inhibiting transcription with ActD. If localization of the AUBPs, indeed, affects the stability of mRNA, results from ActD chase studies have to be interpreted with great care. Reporter systems with inducible transcription have to be favored.

BRF1 and TTP are distributed all over the cell. Murata et al. showed nuclear and cytoplasmic localization of TTP in COS7 cells. Nuclear export was CRM1 dependent and a nuclear localization signal (NLS) could be mapped to the region between the two zinc fingers (Murata *et al.*, 2002). Also for BRF1 and BRF2 CRM1 dependent shuttling was reported (Phillips *et al.*, 2002). The authors of this publication confirmed the presence of the NLS and in addition identified a nuclear export signal (NES at the C-terminus of both BRF1 and TTP. The two reports differ in the point that in 293 cells TTP and BRF1 localized predominantly to the cytoplasm. Seemingly the localization is different in different cell types. In NIH3T3 cells the two proteins are distributed throughout the cell with some variations (Figure 25). The reason for these variations could not be figured out. The presence of a NLS and a NES in BRF1 and TTP indicates that shuttling is relevant for the function of these proteins.

PI3-K stabilizes ARE-mRNA via inactivation of BRF1 and export of HuR. Interestingly, the two stabilizing proteins investigated here (HuR and $AUF1_{p37}$) are stored in the nucleus under conditions of ARE-mRNA decay, whereas substantial levels of the two destabilizing proteins (BRF1 and TTP) are found in the cytoplasm. These findings support the hypothesis that ARE-mRNA is targeted for rapid, exosomal turnover by BRF1 and/or TTP (Figure 31A). Upon stimulation, BRF1 and/or TTP are inactivated by phosphorylation, and/or HuR and AUF1 are exported into the cytoplasm, where they compete with the destabilizing factors for RNA binding and protect the RNA (Figure 31B). PI3-K seems to play an important role in the coordination of this process. As lined out in this thesis, activation of PI3-K, on one hand, promotes export of stabilizing HuR protein, probably via PKC, and on the other hand inactivates BRF1 by phosphorylating S92. Maybe, high concentrations of HuR are sufficient to replace the inactivated BRF1 from the mRNA and protect it from degradation. Another possibility would be that inactive BRF1 and HuR bind together to one ARE, blocking the access of the exosome to the RNA. The fate of the ARE-mRNA is, therefore, determined by the activation status of the AUBPs as well as by the ratio of their local concentration.

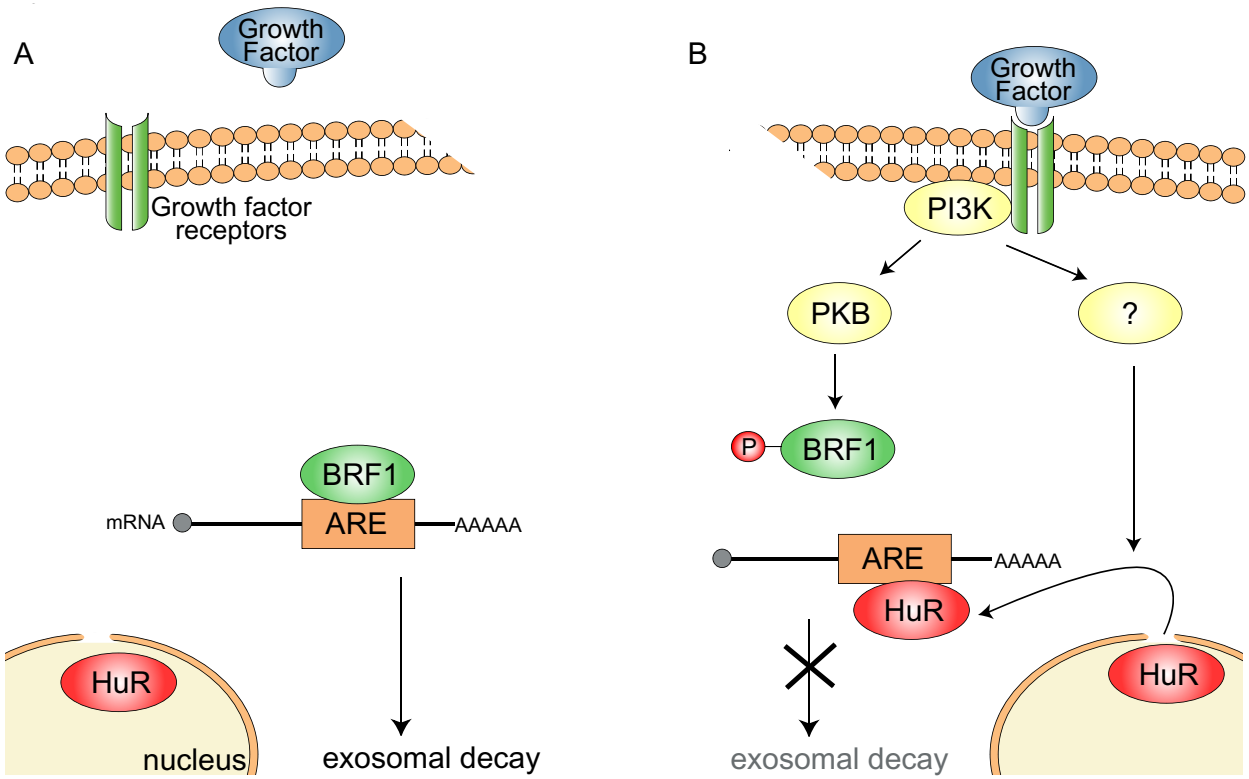


Figure 31: The PI3-K signaling pathway controls ARE-mRNA stability via inactivation of destabilizing factors (BRF1) and activation of stabilizing factors (export of HuR)

Additional means of AUBP regulation. Two aspects of AUBP control have be investigated in this thesis: Localization and inactivation by binding of 14-3-3 in a phosphorylation dependent manner. In addition to that, also other ways of ARE-mRNA regulation have to be taken in account, including the turnover of AUBP mRNA and proteins. TTP was shown independently by two groups to interact with the 3'UTR of its own mRNA and promote degradation of its own message (Brooks *et al.*, 2004; Tchen *et al.*, 2004). Maybe, also other AUBPs are regulated via mRNA turnover. Recent data

in our lab (Don Benjamin, unpublished data) indicate, that BRF1 protein stability is controlled by phosphorylation, displaying another way to regulate mRNA turnover.
Apart from the nucleo-cytoplasmic shuttling also other changes in AUBP localization may be of importance for their function. Upon oxidative stress several AUBPs, including HuR, TIA1, TIA-R, BRF1 and TTP, translocate into stress granules, where translationally silenced mRNA is stored (Kedersha *et al.*, 1999; Kedersha *et al.*, 2000; Stoecklin *et al.*, 2004). Maybe, also other complexes exist, where AUBPs are stored in an inactive state, waiting for the appropriate stimuli.

Physiological Role of BRF1

BRF1 was initially cloned as an EGF-inducible and butyrate-repressible gene (Gomperts *et al.*, 1990). It was then found to bind and destabilize ARE-mRNA. However, it was not known, which ARE-mRNAs are targeted by BRF1 and what the physiological significance of BRF1 is.
Many cyclin and growth factor mRNAs contain AREs in their 3'UTR, and as HuR has been shown to control stability of cyclin mRNA in a cell cycle dependent manner (Wang *et al.*, 2000), it seemed plausible, that the destabilizing BRF1 protein contributes to the rapid turnover of cell cycle regulating factors. Using a NIH3T3 cell line with doxycycline (dox)-repressible BRF1 expression, the role of BRF1 in cell cycle progression was investigated. Indeed, overexpression of BRF1 in these cells did accelerate the progression of serum starved, G0 arrested cells into S phase compared to cells with normal BRF1 levels. Downregulation of BRF1 by siRNA had the opposite effect. One way to explain this phenomena is that BRF1 destabilizes mRNA of cell cycle inhibitors (such as the cyclin-dependent kinase (CDK) inhibitors p15 (containing 3 AUUUA pentamers), p16 (2 pentamers), p21 (2 pentamers) or p27 (7 pentamers); for review see (Sherr and Roberts, 1999)). Yet, the observed effect was reproducible but minimal and it seemed that BRF1 either does not directly regulate stability of one of the key players in cell cycle control or that BRF1 is only one component that acts in concert with other factors, such as TTP or HuR. In both cases, changing levels of BRF1 would only have a marginal effect on the cycling behavior of the cells.
Further insight into the physiological role of BRF1 was gained by work from Ines Raineri and coworkers (unpublished data) in our lab. Mouse embryonic stem cells (ES cells) can be cultured as pluripotent, self-renewing cells in the presence of Leukemia inhibitory factor (LIF). Upon removal of LIF, differentiation is induced. The cells, previously growing as compact colonies, start to spread out and differentiate into various cell types. Differentiation is accompanied by a prominent downregulation of BRF1 mRNA and protein. TTP mRNA levels, on the other hand, increase upon differentiation. Further, downregulation of endogenous BRF1 by siRNA induces a differentiation-like phenotype of ES cells in the presence of LIF. Upon differentiation, cell cycle control of ES cells changes completely (Burdon *et al.*, 2002). The fast cycling, self-renewing cells change to a slower cycle. This correlates with BRF1 downregulation in NIH3T3 cells, where cell cycle progression also seems to be slowed down. However, the data obtained from this set of experiments provide only a vague indication that BRF1 is a player in cell proliferation and no conclusion on any involved mechanisms can be drawn.

Outlook

The work presented in this study provides insight into the mechanisms regulating ARE-mRNA turnover via AUBPs. Progress has been made in understanding the mode of action of BRF1, but still many questions remain unanswered and will be addressed in the future. The model presented in Figure 30 will have to be evaluated in respect to the nature of the different kinases, phosphorylating BRF1 and the functional significance of these phosphorylation events. Is the affinity of doubly (S92, S203) phosphorylated BRF1 to 14-3-3 higher? Does 14-3-3 binding inhibit exosomal targeting of ARE-mRNA by BRF1? Which kinase(s) do(es) phosphorylate S203. Are the other putative phosphorylation sites real? What is their functional significance? By further characterizing the regulation of BRF1, hopefully, we will learn more about the complex process of ARE-mRNA turnover.

Materials and Methods

Primers

Primers for Sequencing

M2135: 5'- ACA AGC TTC CAT GAC CGA G -3'	(pac:5' (HindIII))
M2136: 5'- GGG CGG CCG CTC CTT TC -3'	(pac:3' (NotI)
M2188: 5'- CCC TCG CCC TCG ATC TC -3'	(SPred reverse)
M2189: 5'- GGG CGG TAG GCG TGT ACG -3'	(SPCMV forward)
M2336: 5'- GGC GTG TAC GGT GGG AG -3'	(pminCMV forward)
M2337: 5'- GGA GAC AAT GGT TGT C –3'	(pTRE-myc poly(A) reverse)
TV334: 5'- GCG GTA GGC GTG TAC GGT GGG AGG –3'	(bsdhis-BRF1 forward)
TV335: 5'- CTG CCT GCT GGG GAG CCT GGG GAC –3'	(bsdhis-BRF1 reverse)

Primers for Northern Probes:

TV359: 5'- ACA GAT GTG ACA AGC CAA GG -3'	(ratVEGF)
TV360: 5'- GGT GTG TCT ATA GGA ATC C -3'	(ratVEGF)
M1170: 5'- ACT GTG TTG GCA TAG AGG TC -3'	(murine β-actin)
M1171: 5'- ACC TCA AAG AGA AGC TGT GC -3'	(murine β-actin)
M2352: 5'- TGA TGG TAC ATG CAA GGT GC -3'	(humanGAPDH)
M2353: 5'- ACA GTC CAT GCC ATC ACT GC -3'	(humanGAPDH)

Primers for Mutagenesis

TV277: 5'- GCC GAG TCC CCT CAC ATG TTT G -3'	(huBRF1 S283A forward)
TV278: 5'- CAT GGG CCG GAA GAG GAA GGT G -3'	(huBRF1 S283A reverse)
TV419: 5'- AGG AAT GCT GGA GGC GGG GAC GG -3'	(huBRF1 S203A reverse)
TV420: 5'- TTG CCT TTG CTG GGT TTC CCA GTG C -3'	(huBRF1 S203A forward)

Plasmids

bsd-HisBRF1wt has been described previously (Stoecklin *et al.*, 2002).

bsd-HisBRF1S90/92A (bsd-HisBRF1$_{AA}$) is described in (Schmidlin *et al.*, 2004).

bsd-HisBRF1S203A was obtained by site directed mutagenesis of bsd-HisBRF1wt, using the primers TV419/420

bsd-HisBRF1S283A was obtained by site directed mutagenesis of bsd-HisBRF1wt, using the primers TV277/278.

pTet-Off, pTRE-myc were purchased from Clontech.

pTRE-myc-GFP was constructed by introducing the AflII(blunt)-BfrI(blunt) fragment from MO-GFP (containing GFP under the control of a Molony promoter and a SV40 poly(A) sequence) into the AatII(blunt) site of pTRE-myc.

pTRE-BRF1 contains a XbaI-KpnI(blunt) fragment from bsd-HisBRF1wt in the XbaI-SalI(blunt)site of pTRE-myc-GFP (Stoecklin *et al.*, 2002). See Figure 32A.

pTRE-BRF1S90/92A contains a XbaI-KpnI(blunt) fragment from bsd-HisBRF1S90/92A in the XbaI-SalI(blunt)site of pTRE-myc-GFP (Stoecklin *et al.*, 2002).

pTRE-TTP was constructed by introducing the XbaI(blunt)-HindIII(blunt) fragment of mTTP.tag (Stoecklin *et al.*, 2000) into the HindIII(blunt) site of pTRE-myc-GFP. See Figure 32B.

pcDNA3.1-HuR-HIS/myc has been described in (Ming *et al.*, 2001).

pHuR-green: The EcoRV/EcoRV fragment of pcDNA3.1-HuR-HIS/myc (Ming *et al.*, 2001) was ligated into the EcoRV/SalI(blunt) cut pEGFP-N1 vector (Clontech). See Figure 33A.

pmAUF1-MycHis: was generated by introducing a HindII-EcoRV of mAUF1 into the respective site of pcDNA3.1/myc-HisB (Invitrogen).

pAUF1-green: The HindIII-EcoRV fragment from pmAUF1-mycHis (Ming) was ligated into the HindIII-SalI(blunt) cut pEGFP-N1 vector (Clontech). See Figure 33B.

pSRL was generated from pTet-BBB-ARE^{GMCSF} (Xu *et al.*, 1998) by exchanging the blunted BamHI-BstXI fragment for the blunted BamHI-SphI fragment of pMXh-β-IL3(UTR)wt (Ming *et al.*, 2001).

rasV12: pDCR-HA-rasV12 (Verheijen *et al.*, 1999) was kindly provided by G. Radziwill and J.L. Bos.

RafCT: pcDNA3-Flag-rafCT (Heinrich *et al*. 2000) was kindly provided by K.Moelling.

PI3-K: pEF-Bos-rCD2-p110 was generously provided by D. A. Cantrell (Reif *et al.*, 1997; Reif *et al.*, 1996).

m/pPKB: pECE.p-HA-PKBα (Andjelkovic *et al.*, 1997) was kindly provided by B. A. Hemmings.

MEK6DD: SRα3-MEK6DD (Stein *et al.*, 1996) was generously provided by M. Karin.

p38-AGF: pCMV-p38-AGF (Raingeaud *et al.*, 1995) was provided by R.J. Davis.

pMX-puro was constructed by inserting a HindIII/NotI PCR fragment (primers M2136/M2135) containing the puro sequence from pBABE-puro (Morgenstern and Land, 1990) into the respective sites from the pMX backbone kindly provided by T. Kitamura.

Transformation of *E.coli* ($CaCl_2$-method)

DH5α E.coli cells were grown in 80ml LB medium to OD_{600} of 0.4-0.5. Cells were harvested by centrifugation at 2'500g and the pellet was resuspended in 50ml ice-cold 70mM $CaCl_2$. Cells were incubated for 30min. Again cells were spinned down and resuspended in 2ml 70mM $CaCl_2$ and 2ml 50% glycerol. 100µl aliquots were stored at –70°C. For transformation the competent *E.coli* were thawed on ice. Then DNA (40-100ng) was added and put on ice for 25min. After 2min heat-shock at 42°C, the mixture was incubated for 1h in 1ml LB at 37°C. Then the cells were centrifuged, resuspended in 100µl LB and streaked on selective LB plates.

Isolation of Plasmid DNA

Plasmid DNA was purified from DH5α *E.coli* strains using Plasmid Mini or Maxi kits® from QIAGEN according to the manufacturers protocol. DNA fragments from restriction digests or poly chain reactions (PCRs) were separated on agarose TAE-gel (Percentage dependent on the fragment size from 0.8 to 1.2%). The gel slices of interest were cut out and DNA was extracted using the MinElute Gel Extraction Kit® from QIAGEN. Alternatively DNA was purified using the MinElute Reaction CleanUp Kit®.

DNA Cloning Techniques

Restriction enzyme digests and polymerase chain reactions were performed, using enzymes from Boehringer Mannheim, Roche or New England Biolabs (NEB). The protocols and buffers were adapted according to the conditions suggested by the suppliers.

Site Directed Mutagenesis

For site directed mutagenesis two back-to-back primers were designed containing the respective mutations. PCR was performed with TV277/TV278 (mutation of S283) or TV419/TV420 (mutation of S203) as primers using Platinum® Pfx DNA polymerase (Invitrogen). The reaction was digested with DpnI to specifically remove the methylated bacterial wild type template. The product was purified over a TAE-agarose gel. The excised fragment was then purified using the QIAquick Gel Extraction Kit® (QIAGEN) and ligated, before transformation into DH5α *E.coli*. The presence of the mutation was checked by sequencing and by restriction digest (for S283, where a BSMI was introduced by the PCR reaction).

Sequencing

Constructs were sequenced using the ABI Prism 310 Genetic Analyzer (Applied Biosystems)

or the CEQ 8000 (Beckman Coulter). The sequencing reaction was performed according to the manufacturers instructions.

Cell Culture and Transfection

Cell Lines

NIH3T3: Mouse fibroblast cells were grown in M2 medium (Iscove's Modified Dulbecco Medium (IMDM) supplemented with 10% fetal calf serum (FCS), 50μM 2-mercaptoethanol, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin).

B2A2 cl23: NIH3T3 clone, expressing the tTA transactivator (Xu *et al.*, 1998), was cultured as NIH3T3 cells.

BRF1off11 / BRF1off14: Two puromycin resistant B2A2 clones, stably expressing myc-BRF1 under the control of a tetracycline repressible CMV promoter, were grown in M2 supplemented with 2μg/ml doxycycline.

HT1080: Human fibrosarcoma cells were grown in M2.

RAW 264.7: Mouse macrophages were also grown in M2 medium.

HIRc-B: Rat fibroblast cells, overexpressing the human insulin receptor (kindly provided by P. Blackshear (McClain *et al*., 1987)), were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FCS, 2-mercaptoethanol, glutamine, penicillin and streptomycin as in M2.

MEF-PKBα-/- / **MEF-PKBα+/+**: PKB knockout mouse embryonic fibroblasts and the corresponding wt cells (kindly provided by B. Hemmings (Yang *et al.*, 2003))were also grown in the same medium described for HIRc-B cells.

Transfection

Plasmids and siRNAs were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturers instructions.

Selection

For stable transfection of pTRE-BRF1wt into B2A2 cl23, pMX-puro was cotransfected and positive clones were selected by treating the cells with 2μg/ml puromycin. Clones, expressing the protein of interest were selected by Western blot.

RNAi

siRNA:
BRF1 (mmBRF1): caa gau gcu caa cua cag c (nucleotides 130-148, NM_007564.1)
β-globin (hu β-globin2): caa gaa agu gcu cgg ugc c (nucleotides 245-263, V00497.1)

Immunofluorescence

Cells were grown on cover slips (LabTek ChamberSlides, Nunc) pretreated with 0,1% gelatin. Then the cells were fixed with 4% paraformaldehyde for 10 min. at RT before permeabilizing in Methanol or 0.1% Triton X100 for 2 min. Primary and secondary antibodies were added, diluted in 50µl M2 medium, serving as blocking reagent. Incubation with the antibodies lasted about 15 minutes depending on the antibody. Between the different incubation steps the cells were washed intensively with PBS supplemented with Ca^{2+} and Mg^{2+}. After the final washing step the cells were mounted with Fluoromount-G® (Southern Biotechnology Associates, Inc.) and a coverslip. Cells were analyzed under the fluorescence-microscope (Nikon) with the appropriate filters. Pictures were taken and prepared using the OpenLab software (Improvision)

Western Blot

Approximately $2.5x10^6$ cells were grown to 80% confluency. Medium was poured off and the cells were washed with PBS. At 4°C, 200µl M-PER® Mammalian Protein Extraction Reagent (Pierce) containing 1x Phosphatase inhibitor cocktails 1 and 2 (Sigma) was added to the plate for lysis. After 5 minutes the cells were scraped off and transferred into Eppendorf tubes. The solution was vortexed and then centrifuged at 4°C. Supernatant was taken and protein concentration was measured using Bradford protein assay (BioRad). 30µg of total protein were denatured at 95°C for 2 minutes with sample loading buffer (Pierce) containing 1mM DTT before samples were loaded on 4-20% gradient SDS-polyacrylamide-gels (Anamed). Protein was blotted on Immobilon-P (Milipore) membrane and blocked for 1h in TBS containing 2% ECL Advance blocking reagent (Amersham) and 0.1% Tween-20 (TBS-T). The membrane was incubated over night at 4°C with the appropriate antibody diluted in TBS-T with blocking reagent. Before addition of the secondary antibody (pre-warmed to 37°C) for 45 minutes, the membrane was washed in TBS-T. After final washing, proteins of interest were visualized according to manufacturers protocol using CDP-star (Roche) or ECL advance (Amersham) depending on the secondary antibody and subsequent autoradiography.

Antibodies

Phospho-PKB (Ser473): mouse monoclonal IgG2b (clone 587F11), Cell Signaling Technology;
PKB: rabbit polyclonal, Cell Signaling Technology (#9272);
Phospho-p44/42 (Thr202/Tyr204) (Erk1/2): mouse monoclonal IgG1(clone E10), Cell Signaling Technology;
p44/42 MAP Kinase: rabbit polyclonal, Cell Signaling Technology (#9102);
Phospho-p38 MAPK: rabbit polyclonal (Thr180/Tyr182), Cell Signaling Technology (#9211);
p38-MAPK: rabbit polyclonal, Cell Signaling Technology (#9212);
HuR: mouse monoclonal (clone 3A2), Santa Cruz;
Xpress: mouse monoclonal IgG1, Invitrogen;
α-tubulin: mouse monoclonal IgG1 (clone 236-10501), Molecular Probes;
myc: monoclonal mouse (clone 9E10);
Secondary antibodies: Alexa-Fluor® 555 goat anti-mouse IgG(H+L); AP-coupled goat anti rabbit IgG(H+L) (Southern Biotech); HRP coupled goat anti mouse IgG (Santa Cruz).

Anti-BRF1/Anti-Phospho-BRF1

Anti-phospho-BRF1 and anti-BRF1 antibodies were generated by immunizing rabbits with the KLH-linked peptides F-R-D-R-S-F-S(PO_3H_2)-E-G-G-E-R-L (aa86 - aa98 of BRF1) and S-D-Q-E-G-Y-L-S-S-S-S-S-S-H-S-G-S-D-S-P-T (amino acids 300-320) (Neosystem). The immune response of the animal was boosted twice in two-week intervals. One week after the second boost the rabbit was bled and the sera were tested for their ability to recognize recombinant (*in vitro* phosphorylated for the phospho-antibody) BRF1 on western blot. Positive sera were affinity-purified using the peptides mentioned above linked to activated CH Sepharose 4B (Pharmacia Biotech) according to the manufacturer's protocol.

λ-Protein Phosphatase (λ-PPase)-Treatment

Cells were lysed as described before, without PPase inhibitors. The extract was supplemented with 2mM MnCl. 10units of λ-PPase were added for indicated amounts of time at 30°C. The reaction was stopped by the addition of sample loading buffer and denaturation at 95°C.

In vitro Phosphorylation

The reaction was performed in 30mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 1mM DTT, 0.2mM ATPγS (unlabelled or P^{32}-labelled) with 4ng/µl rBRF1 and 10ng/µl activated PKB (kindly provided by B.A. Hemmings) for 30' at 30°C (Yang *et al.*, 2002).

Northern Blot Analysis

RNA Isolation

1-5x10^6 cells were grown to sub-confluency in 10cm culture dishes. For ActinomycinD (ActD) chase experiments ActD was added at a concentration of 5µg/ml. After removal of the medium, cells were washed with PBS and subsequently RNA was extracted using the method described by N. Gough (Gough, 1988).

Electrophoreses and Blotting

20-50µg RNA were resolved on 1.1% agarose/formaldehyde gels in MOPS buffer (10mM MOPS (pH 7.0), 8mM Sodium acetate and 1mM EDTA (pH 8)) and blotted onto Highbond N+ membranes (Amersham) overnight with 20xSSC (1xSSC contains 150mM NaCl and 15mM Na-citrate (pH 7.0)). The membrane was prehybridized at 55°C (RNA probe) or 65°C (DNA probe) in corresponding hybridization buffer, before addition of the probe.

^{32}P-Labelled RNA Probe

For SP6 *in vitro* transcription 1,5mM ATP, CTP, UTP, 1,25 µM GTP, 2,5mM DTT, 0,05mg/ml BSA (NEB), transcription buffer (Promega) linearized template DNA (amount dependent on the probe), 1U/µl SP6 polymerase (Promega) and 10µCi α^{32}P-GTP were mixed and incubated at 37°C for 1hr. The probe was then phenol/chloroform extracted followed by ethanol precipitation. The probe was denatured at 95°C for 10min prior to addition to the blot. The blot was hybridized for 16h in hybridization solution containing 50% (v/v) Formamide (Intergen Company). 5xSSC, 5x Denhardt's solution (1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (Sigma, Fraction V)), 10ml PIPES (pH 6.4), 0.4mg/ml yeast RNA, 0.5% SDS and 5mM EDTA (pH 8.0). Then, it was washed with 2xSSC/0.2%SDS, followed by 0.2xSSC/0.2%SDS and

0.1xSSC/0.2%SDS for 30min each at 65°C. Then, the radioactive signal was visualized using a Phosphor-Imager (Molecular Dynamics) with the Quantity One software (BioRad).
For detection of IL3-3'UTR mRNA, an XbaI-EcoRI fragment of murine cDNA was used as a template for *in vitro* transcription. The template for the actin probe was a 260bp fragment of murine β-actin generated by RT-PCR using M1170 and M1171 as primers.

^{32}P-Labelled DNA Probe
For DNA probes, 25-50ng template, and 2pmol/µl primer in a total volume of 10µl were denatured at 95°C for 5min. Then 1x EcoPol reaction buffer (BioLabs), 0.5mM dNTPs (lacking dCTP), 10µCi α^{32}P-dCTP and 5U Klenov polymerase (exo- from BioLabs) were added and the mixture was incubated for 1h at 37°C. After purification over the QIAquick purification kit (QIAGEN) columns, the probe was denatured at 95°C for 5min and then added to the hybridization reaction. The hybridization solution for DNA probes contains: 6.8%SDS, 0.5M Na/PO_4 (pH 7.2), 1% BSA (Sigma, Fraction V) and 2mM EDTA. The blot was hybridized overnight at 65°C and then, washed with 2xSSC/0.2%SDS and 0.2xSSC/0.2%SDS for 15 min each at 50°C. The radioactive signal was visualized using a Phosphor-Imager (Molecular Dynamics) with the Quantity One software (BioRad). For detection of ratVEGF mRNA, a 353nt long portion of the cDNA was amplified by PCR with primers TV359 and TV360, and used as a template for a ^{32}P-dCTP labelled, TV360-primed DNA probe. For the GAPDH probe, a fragment of human GAPDH was amplified by RT-PCR using the M2352/M2353 primer pair was used as a template for the M2352 primed ^{32}P-dCTP labelled DNA probe.

Stripping
The blot was washed with boiling 0.5% SDS and prehybridized as described above, before addition of the new probe.

Propidium Iodide Staining
Cells were trypsinized and washed with PBS. After spinning them down, they were resuspended in PI-staining solution (50µg/ml propidium iodide, 0,1% tri-sodium citrate, 0,1% Triton X-100, 0,2mg/ml RNase A) on ice. The samples were analyzed by flow cytometry using a red filter (580nm).

Methyl-^{3}H-Thymidine Incorporation
Cells were grown in 96 well plates, serum-starved for 24h, then, FCS was added, 2h after addition of 0,5 µCi Methyl-^{3}H-Thymidine/well. Cells were transferred to a filter paper and washed using the cell harvester from Inotech, then, incorporation of radioactivity was measured by a beta-counter (Packard).

Flow Cytometry
Samples were analyzed using a FACScan (Beckton Dickinson) and Cellquest software.

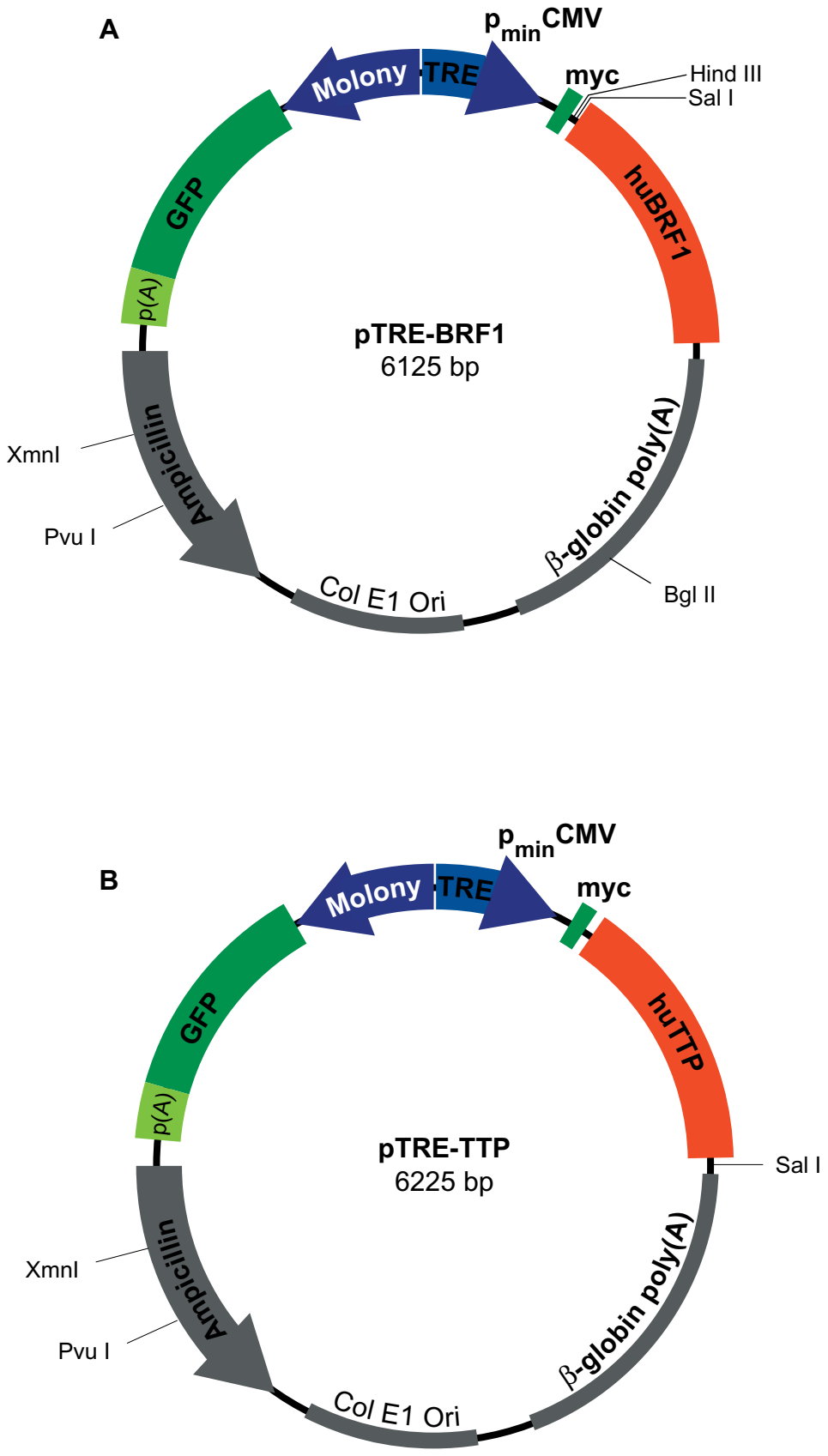


Figure 32: Map of pTRE-BRF1 and pTRE-TTP, allowing tetracycline controllable expressing of myc-tagged proteins. For selection, a GFP gene and an ampicillin resistance are located on the same plasmid. Restriction enzymes, cutting only once, are shown.

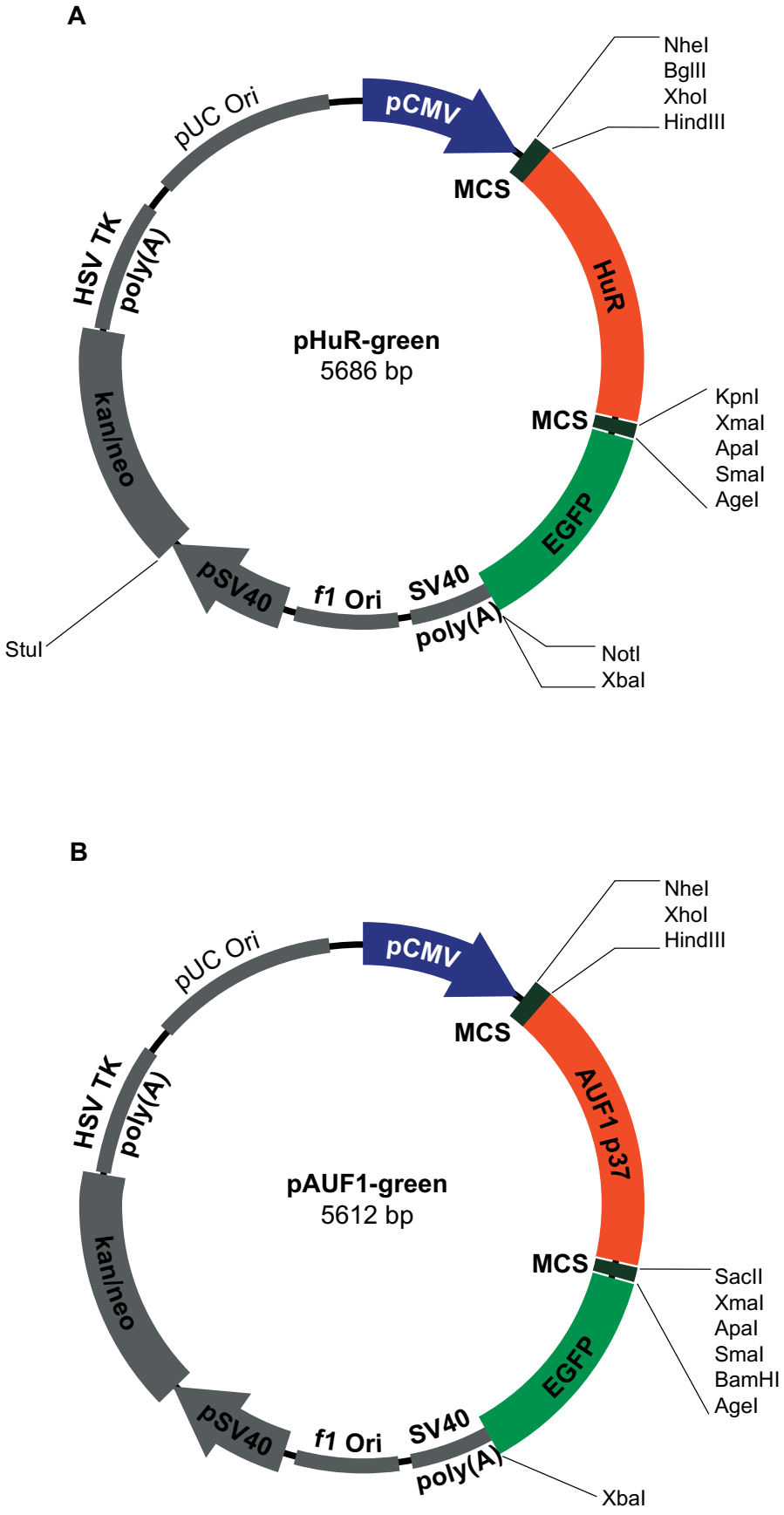


Figure 33: Map of pHuR-green and pAUF1-green, expressing C-terminal GFP fusion proteins. In addition a kanamycin resistance gene is located on the plasmids. Restriction enzymes, cutting only once, are shown.

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Figures and Tables

Figures

Tables

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Declaration

I declare that I wrote this thesis “Regulation of mRNA Stability via BRF1 and other AU-Binding Proteins” with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university.

Martin Schmidlin-Stalder